ALLOREACTIVE LYMPHOID CELLS INFILTRATING CARDIAC ALLOGRAFTS

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Alloreactieve lymfocytaire infiltraten in het harttransplantaat

PROEFSCHRIFT

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Uitgeverij Eburon
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2601 CW Delft

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CONTENTS

LIST OF ABBREVIATIONS

B-LCL	B lymphoblastoid cell line
$ß_2M$	$ß_2$ microglobulin
CML	cell mediated lympholysis
CsA	cyclosporine-A
CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T lymphocyte precursor
EBV	Epstein-Barr Virus
EMB	endomyocardial biopsy
FACScan	Fluorescence Activated Cell Scan
H-ALG	horse-anti lymphocyte globulin
HLA	Human Leucocyte Antigen
IL-2	interleukin-2
MHC	Major Histocompatibility Complex
NK cell	natural killer cell
PBMC	peripheral blood mononuclear cells
PHA	phytohaemagglutinin
TCR	T cell receptor

CHAPTER 1

INTRODUCTION

Clinical heart transplantation

Experimental heart transplantation has been performed in animal models since the beginning of this century in several centres throughout the world (1,2). In Rotterdam such studies have been performed since the seventies. In these studies, heterotopic and orthotopic transplantations were compared in dogs, and the development of accelerated coronary artery disease in allograft recipients was studied (3-5). Clinical transplantation has become possible after several technical break throughs: the discovery of extracorporeal hypothermia in 1948, and the development of the heart-lung machine (6). It then became possible to develop surgical techniques which allowed intrathoracic, orthotopic, cardiac allografting (7). The first technically successful human orthotopic heart transplantation was performed in 1967 by Christian Barnard in South Africa (8). In the following 2 years approximately 150 heart transplantations have been performed throughout the world, with almost invariable discouraging results, due to high mortality rates caused by infections and severe rejection in the early postoperative period. For this reason, most heart transplantation programs were discontinued. Since 1978 the results have much improved, mainly because of the development of reliable methods for the detection of rejection using endomyocardial biopsy (9), and advances in immunosuppression with the

introduction of cyclosporine (10). Using these new methods, a 5 year graft survival of 50% was reported by the Stanford group in 1982 (11).
Between 1980 and 1991 over 21,000 heart transplants have been performed worldwide, with actuarial survival rates of 78%, 67% and 52% at 1, 5 and 10 years, respectively (12). In the Netherlands, the first human heart transplantation was performed in 1984 in Rotterdam. During the first 9 years of the Rotterdam heart transplant program, more than 200 patients received a cardiac allograft (13,14). The patient survival rates after 1 month, 1 year, 5 years and 7 years are 96%, 92%, 84% and 77% respectively. Graft rejection is still a major cause af graft dysfunction and failure. In Rotterdam, approximately 75% of the patients experience one or more acute rejection episodes during the first posttransplant year. Rejection results from cellular and humoral responses against foreign antigens, provoked by the genetic disparity between host and donor. Of the foreign antigens that may be recognized by the immune system the human major histocompatibility complex is the most important (15-17), but rejections directed towards non-MHC antigens have also been reported (18-21). In contrast to renal and bone marrow transplantation, donors and recipients of cardiac allografts are not matched for HLA antigens, because the HLA matching tests are too time consuming, as the maximally tolerated ischaemia time of the explanted heart is only 4 hours (22).

The human major histocompatibility complex (MHC)
The human MHC molecules, termed HLA (human leucocyte antigen) molecules, are highly polymorphic membrane glycoproteins found on the surface of nearly all nucleated cells. They are encoded by closely linked loci on the short arm of chromosome 6 (23). Class I molecules (HLA-A, B, C in humans) are composed of a heavy peptide chain of 43 kDa, non-covalently linked to a smaller 11 kDa peptide called $ß_2$ microglobulin ($ß_2M$). The extracellular part of the heavy chain is organized into three globular domains (α_1, α_2 and α_3; figure 1). With a few exceptions, class I heavy chains seem to need both $ß_2M$ and a tightly bound peptide for their conformational integrity (24-26). Townsend et al. (24) showed that the nature of the bound peptide has significant consequences for the final conformation and function of the MHC molecule. The α_3 domain and $ß_2M$ are relatively conserved and show amino-acid sequence homology to immunoglobulin constant domains. The α_1 and α_2 domains are most polymorphic. X-ray crystallographic analysis of the HLA-A2 molecule revealed that the α_1 domain has the same tertiary fold as α_2, while α_3 has the same tertiary fold as $ß_2M$. The domains α_1 and α_2 pair to form a platform of a single eigth-stranded ß-sheet covered by two α helices. The long groove between the helices form the peptide binding site for processed foreign (or self) antigens (27). The most polymorphic residues are positioned along the groove (28), which is located

on the top surface of the molecule. Other functional residues point away from the peptide-binding site or are located outside the groove, and may be important for interaction with a T-cell receptor.
HLA-class II molecules (HLA-DR, DQ, DP) have a more restricted distribution than class I molecules, and are expressed on B cells, antigen-presenting cells such as macrophages, dendritic cells and Langerhans cells, and on activated T cells. They consist of α and ß polypeptide chains of molecular weight 34 kDa and 28 kDa, respectively.
On the basis of the MHC class I structures, a hypothetical model of MHC class II structure has been proposed (29,30). The membrane-proximal α_2 and $ß_2$ domains resemble the immunoglobulin domains, like the α_3 and $ß_2M$ domains of class I molecules (31). The most polymorphic are the α_1 and $ß_1$ domains distal to the cell membrane (32,33). The peptide binding portion of HLA class II molecules has been proposed to be similar to that of MHC class I molecules (30).

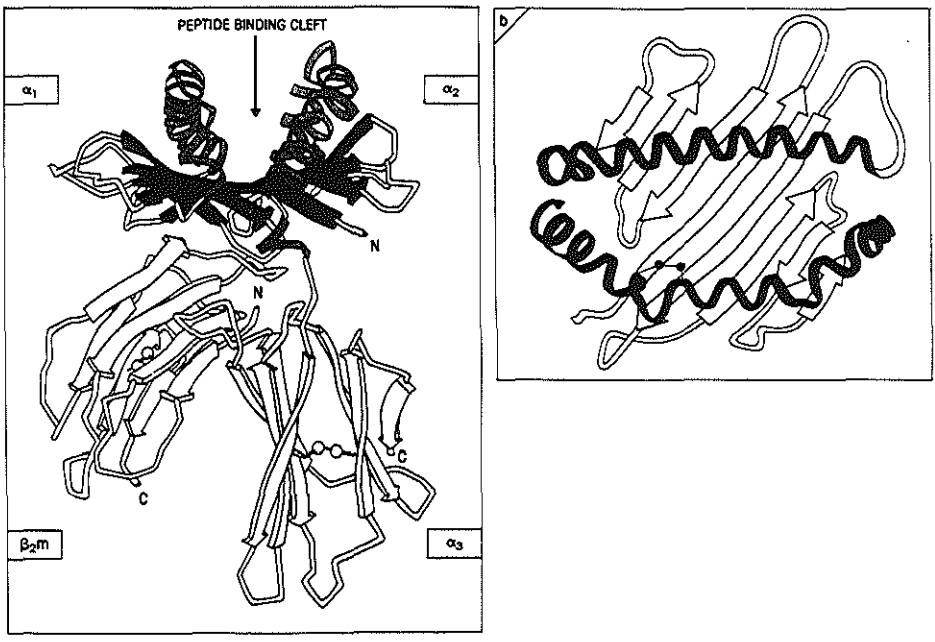

Figure 1 Schematic representation of the crystallographic structure of an HLA class I molecule. Left: side view of the class I molecule with the typical folding of the polymorphic α_1 and α_2 domains at the top, and the α_3 domain and $ß_2M$ at the bottom. Right: Top surface of the α_1 and α_2 domains showing the groove formed by the two α helices, which form the peptide binding site for processed foreign or self antigens.

T lymphocytes and the T cell receptor

T and B lymphocytes are the cells responsible for specific immunity. Both populations of cells possess a membrane-bound antigen receptor which mediates the specific response of a given T or B cell to a particular antigen. The antigen receptor of B lymphocytes is a membrane bound immune globulin of the IgM class, which recognizes conformational determinants on protein antigens. Meuer et al. (34) provided evidence that the T cell antigen receptor (TCR) consists of a disulphide-linked heterodimeric glycoprotein composed of α and ß chains, each with a molecular weight of approximately 45 kDa. This TCR recognizes short peptides from processed antigens presented by antigen presenting cells and other cells, as a complex of antigen derived peptide bound to MHC molecules on the cell surface (reviewed by Marrack and Kappler, ref. 35). In contrast, immunoglobulin molecules can bind to both free and membrane-bound antigens. The α and ß chains of the TCR are physically linked with proteins of the CD3 complex, which are involved in signal transduction during T cell activation (36,37). The CD3 structure consists of at least 5 protein chains (38,39). After engagement of the TCR, the CD3 complex transduces a signal to the interior of the cell, inducing a cascade of intracellular reactions that results in the effector function specific for this particular cell. In the eighties, a second set of clonally varying TCR heterodimers has been discovered, consisting of a τ and δ chain (29, 40-42). Cells bearing these receptors appear earlier in ontogeny than αß-TCR bearing T cells. τδ receptors are present on a small percentage of peripheral T cells (1-10%) and thymocytes, and on a majority of dendritic T cells in the skin (43). Furthermore, they have been derived from human cardiac allografts where the presence of these cells appeared to correlate with stable graft function (44) or chronic rejection (45,46). The τδ TCR's seem to play a similar role in T-cell activation as αß TCR's. Most τδ TCR bearing T cells express neither CD4 nor CD8, and show MHC-unrestricted cytotoxicity (47). Also the cardiac allograft derived τδ TCR bearing cells failed to show donor directed cytotoxicity (48).

Activation of T lymphocytes depends not only on the T cell receptor, but also on accessory cell surface glycoproteins like CD4 and CD8 (49,50). The CD8 molecule is associated with the CD3/T cell receptor complex on the surface of cytotoxic lymphocytes, and functions as a coreceptor in the process of binding to the target cell as well as during the subsequent lysis of target cells (51). However, cytotoxic T cell clones with high affinity T cell receptors for antigen do not need CD8 molecules to stabilize antigen binding, contrary to cells with low affinity T cell receptors (52-55). The cytotoxic function of such T cells with high affinity T cell receptors was shown to be resistant to inhibition with CD8 monoclonal antibodies. MacDonald and coworkers (55) found that this phenomenon was most evident in cytotoxic T cells that had been primed in vivo with the appropriate antigens, and he

attributed this to a selection in vivo for clones with a high affinity for the antigen.

MHC-T cell interaction

T cells respond to antigen only when this antigen is presented in association with products of the MHC. This phenomenon was termed MHC-restriction, and involves the requirement of self-MHC for the recognition of antigen (56). There were two different interpretations: 1) that T cells have a single receptor for an combination of antigen and MHC or 2) that T cells have two different receptors, one specific for antigen and a second specific for MHC, both of which have to be occupied to stimulate the T cell bearing them. It is now recognized that the first theory is correct (34, 57-59). The specificity of antigen recognition by T cells is influenced both by the primary sequence of the MHC-bound peptides and the polymorphic residues of the MHC molecules. Two pathways of antigen processing and presentation of antigen to T cells by the two classes of MHC molecules have been distinguished: exogenously introduced soluble antigens (taken up and degraded by antigen presenting cells) generally associate with MHC class II molecules and not with class I (60). For antigens synthesized within the cell itself and degraded in the cytosol and/or the endoplasmatic reticulum (the 'endogenous' route, e.g. viral antigens), the resulting peptides are usually presented by class I molecules (61).

Rejection of organ allografts

The replacement of diseased organs by a transplant of healthy tissue has long been an objective in medicine but has been frustrated by the uncooperative attempts by the body to reject grafts from other individuals. The polymorphic histocompatibility system confers each individual with an antigenically distinct makeup, which results in allograft reactions when the immune system of the graft recipient is in contact with donor tissue. Rejection is a complex process in which cellular and humoral mechanisms are each deployed in response to foreign antigens. As described in this thesis (chapter 5), a higher number of HLA mismatches between donor and recipient is correlated with a lower freedom from rejection and a lower graft survival. In contrast to renal and bone marrow transplantation, donor hearts are randomly allocated to the recipients, because the HLA matching tests are too time consuming, as the maximally tolerated ischaemia time of the explanted heart is only 4 hours (22). This lack of HLA-compatibility of donor and recipient tissues increases the chance of episodes of acute rejection. Fortunately, most rejection episodes can be treated adequately. To minimize the development of rejections, heart transplant recipients generally receive higher doses of immunosuppression than kidney recipients.

Clinically, rejection of the allograft can be devided into four types, based on their time course of appearance:

1. Hyperacute rejection develops within 48 hours after transplantation, and can occur within minutes of allograft revascularization. This type of rejection occurs in individuals with pre-existing humoral antibodies (62,63), either due to blood group incompatibility or presensitization to MHC antigens through blood transfusion, pregnancies or previous transplants. Preformed antibodies react immediately with donor antigens in the graft and bind complement. With the current prospective crossmatching techniques, the incidence of hyperacute rejection is less than 1% in renal transplantation (64). In Rotterdam, this type of rejection has not occurred among the first 200 heart transplantations (13).

2. Accelerated acute rejection may be caused by antibodies and/or cells and occurs within the first 5 days after transplantation. This type of rejection occurs in patients in whom no preformed antibodies have been demonstrated before transplantation. Recipients manifesting this type of rejection probably are high responders for donor antigens. The fast response is generated from immunological memory after a previous encounter with donor-like antigens. In our centre, such a rapidly progressive type of rejection occurred in 3 of 200 heart transplant recipients, and resulted in death of these patients (13).

3. Acute early rejection may occur at any time after transplantation, but is most commonly seen within the first 6 months. In our centre, 75% of patients have experienced one or more acute rejection episodes at six months after transplantation (13,14). Heart transplant recipients frequently do not exhibit clinical signs of acute rejection until the process has advanced to the stage where irreversible damage has occurred. Since histologic rejection usually precedes clinical symptoms, endomyocardial biopsies are performed at regular intervals. Acute rejection is characterized by dense interstitial and/or perivascular cellular infiltration with a predominance of mononuclear cells. In cases of moderate or severe acute rejection, this is accompanied by myocyte necrosis and interstitial edema (65,66).

4. Chronic rejection. This form of rejection is characterized by variable degrees of narrowing of the graft arteries and arterioles, resulting from infiltration of the intima by mononuclear cells, migration and proliferation of vascular smooth muscle cells and fibroblasts from media to intima, and intimal deposition of extracellular matrix material (67-69). This process is initiated by allogeneic cellular immune reactions directed against incompatible transplantation antigens of the graft vasculature. Many locally produced lymphokines play an important role in the maintenance of this process. We found a greater predominance of $CD4^+$ cells in endomyocardial biopsy-derived T cell lines obtained at the time of the diagnosis of chronic rejection (70). This supports the hypothesis of Libby and coworkers (69) that $CD4^+$ helper T cells interacting with foreign HLA class II antigens play an

important role in the etiology of the vascular lesions. The diagnosis of chronic rejection depends largely on visual evaluation of coronary angiographic findings, by which method gradual decreases in luminal diameters are distinguished (71-73). Systematic annual angiographic studies have shown significant vascular lesions in approximately half of the transplants within the first 5 post transplant years (71,74).

Graft infiltrating cells

Even under adequate immunosuppression, organ allografts are usually infiltrated by recipient mononuclear cells that often show donor directed cytotoxicity in in vitro assays. The mere presence of these cells does however not necessarily lead to acute allograft rejection (75-78, this thesis chapter 3), although some investigators have reported a positive correlation between early in vitro growth of graft infiltrating lymphocytes and the presence of acute cellular rejection (77,78).

It is generally recognized that the allospecific T lymphocyte is the primary mediator of allograft rejection (79). This was proven by experiments in neonatally thymectomized mice, who are unable to reject skin grafts. This capacity was restored by injection of lymphocytes from a syngeneic donor. Furthermore, injection of T cells from a donor which has already rejected a graft will result in accelerated rejection of a further graft of the same type. In most studies, the presence of specific cytotoxic cells within a graft has always correlated with tissue rejection.

From quantitative studies of specific donor directed T cells in situ (80,81) it appeared that these cells only make up less than 1% of the total inflammatory population in the graft, although these specific cells are highly concentrated in situ when compared with the recipient lymphoid system or the peripheral blood (80,82). Other, non-specific, inflammatory populations must therefore also be responsible for the allograft damage during rejection. These populations include non-donor directed lymphoid and plasma cells, large granular lymphocytes, natural killer cells and mononuclear phagocytes, and are recruited to the graft by lymphokines produced by activated specific lymphocytes. Natural killer cells have been shown to be among the first cytolytic lymphoid cells to infiltrate the graft during rejection (83,84), while macrophages are the largest cell population by the end of the acute rejection process, and may play a key role in the injury of the graft tissue (85).

The alloreactive response is initiated by antigen presenting cells that present donor antigens to resting T cells, and produce interleukin 1 and other interleukins. Both dendritic cells and endothelial cells are reported to be able to function as antigen presenting cells, although some investigators have shown that endothelial cells are no potent stimulators of resting T lymphocytes (86). Members of the dendritic cell family are present throughout the body in non-lymphoid as well as lymphoid tissues. There is

no consensus as to whether the activation of resting T cells occurs centrally (in lymphoid organs) or peripherally (in the graft). It was found in mice that, after transplantation, donor dendritic lymphocytes migrate rapidly from cardiac allografts into the recipients's spleens, where they associate with $CD4^+$ T cells (87). Larsen et al. (87,88) have postulated that immature cytotoxic T cells are sensitized in the lymphoid organs, and migrate as immature cells to the graft, where they receive their final maturation step by lymphokines. The thus activated antigen-reactive T cells display the interleukin-2 receptor on their surfaces and produce several lymphokines, which in turn results in T cell proliferation, recruitment of other cell types to the graft, and induction and upregulation of MHC and adhesion molecules in the graft, rendering the graft tissue susceptible to attack by specific alloreactive T lymphocytes.

Cytokines play an important regulatory role in the rejection process. Our recent studies using the reverse transcriptase polymerase chain reaction, showed that the expression of interleukin-2 genes, but also of interleukin-4 genes, was significantly higher during acute cardiac allograft rejection when compared to samples taken in periods without rejection (89). This is in agreement with observations of others (90,91) that in murine cardiac allografts genes for IL-2 and IL-4 are induced during rejection.

Immunohistochemical studies in human and animal heart and kidney transplantation and in sponge matrix allograft models have revealed that mononuclear infiltrates in rejecting allografts are dominated by CD4+ and CD8+ lymphocytes, and cells of the monocyte/macrophage lineage (85, 92-94). Biopsies from nonrejecting hearts were shown to contain significantly fewer T cells and other mononuclear leucocytes than rejecting hearts (93-98). During the early phases of rejection, CD4+ cells showed the greatest influx, with subsequent recruitment of cytotoxic cells and macrophages.

The immunohistochemical techniques provide much information about the phenotypic composition of the infiltrates, but do not discriminate between those graft infiltrating lymphocytes that are primed to mediate rejection and other activated cells not relevant to the rejection response, or those present as passive resident lymphocytes. In the seventies and the eighties several methods were described to isolate lymphocytes directly from the graft, in order to study their functional characteristics. This was done by mechanical extraction from rejected human renal allografts (99) or from sponge matrix allografts in mice (101,102), or by enzymatic digestion of rejected allografts (103-105). The cells that were obtained this way have demonstrated allospecificity.

The most recent and efficient method consists of propagation and expansion of activated T lymphocytes directly from human allograft specimens, using culture medium enriched with interleukin-2 (106-108). This method is based upon the knowledge that (allo-) activated T cells have acquired interleukin-2

receptors during activation, and require subsequent interleukin-2 for further propagation (109). This way, only those T cells that have been activated in vivo are supposed to expand selectively.

Aim of the study

As is discussed above, alloreactive T lymphocytes are the initiators and effectors of acute rejection of organ transplants. The aim of this thesis is to describe reactivity patterns of these cytolytic effectors of host origin that infiltrate the cardiac allograft, and to shed some light on actual events of the acute rejection process within the graft. For this purpose we have isolated and characterized T cell lines from human cardiac biopsies, as described in chapter 2. In chapter 3 growth patterns, and phenotypic and functional characteristics of the cell lines are studied in relation to histological rejection grade and time after transplantation the biopsy was taken.

In chapter 4 we investigate the clinical relevance of qualitative and quantitative differences between the characteristics of cytotoxic T lymphocytes infiltrating the grafts of patients with and without acute rejections.

Despite the use of adequate immunosuppressive therapy, the degree of HLA matching between donor and recipient plays an important role in the immune response against a transplanted heart, which is reflected in a higher freedom from rejection and graft survival in donor recipient combinations with fewer HLA mismatches. In chapter 5 the effect of HLA-A, B and DR mismatches on the functional and phenotypic characteristics of endomyocardial biopsy derived T cell lines is analyzed.

In chapter 6 we show that antilymphocyte immunoglobulins change the specificity of the alloimmune response within the transplanted heart, when compared with patients receiving cyclosporine from the day of transplantation.

Finally, in chapter 7 CTL responses are analyzed in patients with cytomegalovirus infection. This study shows that especially in patients with both rejection and infection donor directed cytotoxicity is increased, while this is not the case in patients with only one of these complications.

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CHAPTER 2

MATERIAL AND METHODS

Patients

From june 1984 until the end of 1993 over 200 heart transplantations have been performed. The patient survival rates at 1 month, 1 year, 5 years and 7 years are 96%, 92%, 84% and 77% respectively. Malignancy, graft coronary disease and rejection were the main causes of death (1). Early immunosuppressive prophylaxis consisted of intravenous cyclosporin (Sandimmune^{R}, Sandoz, Basel, Switzerland), polyclonal anti-T cell antibodies (Horse-ATG), monoclonal antibodies against CD3 (OKT3) or monoclonal antibodies against the interleukin-2 receptor (BT563). Most patients had received preoperative blood transfusions and all received cyclosporine and low-dose prednisone as maintenance immunosuppression. With this regimen, 25% of patients remained free from rejection at 6 months after transplantation, and 24% at three years (figure 1). Until 1993, the dose of cyclosporin was adjusted according to specific plasma-through levels, and whole blood measurements were employed thereafter. The mean number of mismatches between donor and recipient was 1.25, 1.62 and 1.40 for HLA-A, B, and DR, respectively. Rejection was monitored by endomyocardial biopsy, as described below. Grading of the biopsies was according to Billinghams criteria of none, mild, moderate and severe rejection (2,3). For the diagnosis of moderate rejection, the coexistence of myocyte necrosis and

mononuclear infiltrates was required. In that case, anti rejection treatment was instituted, which consisted of bolus steroids or, in case of ongoing rejection, of a two weeks course of a polyclonal rabbit anti thymocyte globulin preparation. Some patients received an additional course of OKT3 for ongoing rejection. There were no cases of severe rejection.

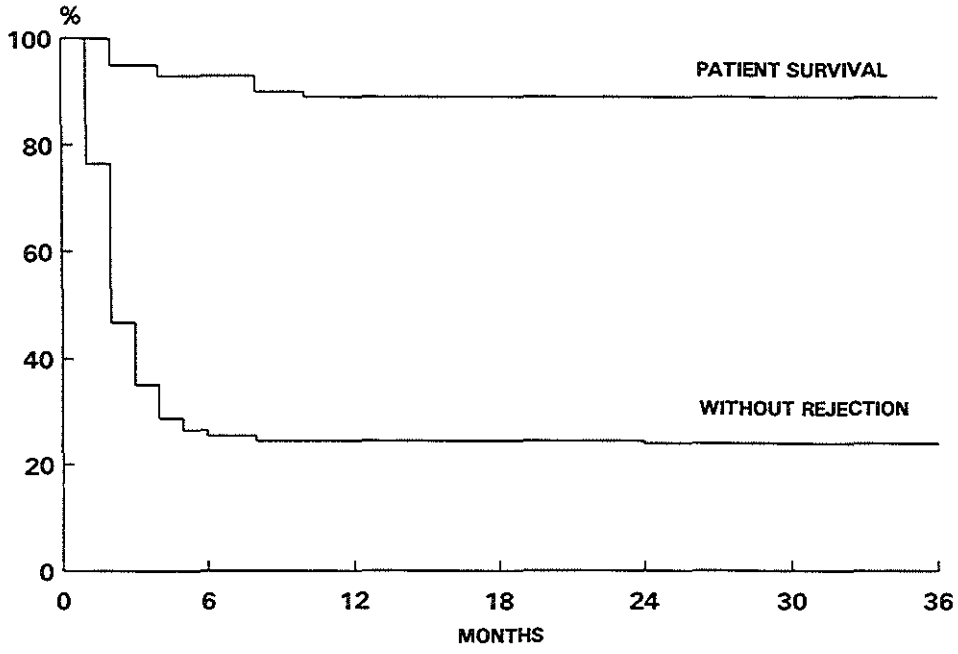

Figure 1 Three year-actuarial patient survival (89%) and freedom from rejection (25%) in 200 heart transplant recipients.

Endomyocardial biopsies

The diagnosis of acute rejection depends on histological evaluation of endomyocardial biopsies. In the early post transplant period serial biopsies were obtained at weekly intervals. Later, endomyocardial biopsies were taken less frequently, declining to once every 4 months at one year. After an acute rejection episode, the next biopsy was taken one week following anti-rejection therapy.

During right ventricular catheterization four or five biopsy samples were obtained. Three or four were used for histologic evaluation, and one was placed in culture medium (RPMI-1640).

Until early 1992 we used conventional Billingham's criteria for assessment of the histological rejection grade (2). These criteria were used in all studies

presented in this thesis: Grade 0: no evidence of rejection, no infiltrate. Grade 1: mild infiltration of mononuclear cells. Endocardial and interstitial edema; diffuse perivascular and endocardial infiltration with pyroninophylic[1] lymphocytes. Grade 2: moderate rejection. Perivascular, endocardial and interstitial infiltrates with pyroninophylic lymphocytes. Focal myocytolysis (necrosis). Grade 3: severe rejection. Vessel wall- and myocyte necrosis with interstitial bleeding. Interstitial infiltrates with polymorphonuclear cells and pyroninophylic lymphocytes. Grade 4: resolving rejection, Active fibrosis, some small, non-pyroninophylic lymphocytes, some plasma cells and haemosiderin.
Anti rejection therapy was considered necessary in cases of 'moderate rejection'.
More recently, the International Society for Heart and Lung Transplantation has proposed a universal grading system for allograft rejection allowing comparison of the results in different centers (3). In our centre, this grading system was used since 1991.

Culture method
Each endomyocardial biopsy was divided into 2 or more fragments and placed into 2 or more wells of a 96 well roundbottom tissue culture plate (Costar, Cambridge, MA) with 200 µl culture medium per well, in the presence of 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Peripheral blood mononuclear cells were isolated by Ficoll-Isopaque (δ = 1.077) density gradient centrifugation. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Half the culture medium was refreshed every 2-3 days. When growth was observed the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing or cell death was observed the cultures were restimulated by adding either 10^5 irradiated (40 Gray) donor spleen cells/well or, when available, 10^4 EBV transformed donor cells/well (irradiated with 80 Gray). Donor cells were rarely (only in approximately 6% of cultures) added before testing the cultures for donor directed cytotoxicity in a cell-mediated cytotoxicity assay.

[1] The methyl green pyronin stain is used to highlight activated lymphocytes.

Allogeneic stimulator/target cells

B lymphoblastoid cell lines originated from infection of fresh peripheral blood mononuclear cells or spleen cells with EBV obtained from the marmoset cell line B95-8 as described by others (4,5). These cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Sera-Lab, Sussex, England).

T lymphoblastoid cell lines were obtained by culturing peripheral blood mononuclear cells or spleen cells for at least 5 days in the presence of 1% PHA-M (Difco, Detroit, MI) and culture medium: RPMI 1640 supplemented with 5% pooled human serum, 5% v/v Lymphocult-T (Biotest).

K562 was cultured in RPMI 1640 with 10% heat-inactivated foetal calf serum.

Phenotypic analysis

Surface differentiation antigens were analysed by two colour flow cytometry after staining with monoclonal antibodies directed against CD3 (anti-leu4) as a pan mature T cell marker, WT31 as a marker for the α/ß chain of the TCR and CD8 (anti-leu2) and CD4 (anti-leu3) as T cell subset markers. CD16 (anti-leu 11) and CD56 (anti-leu 19) were used as markers for NK cells. Antibodies were directly conjugated to fluoresceine (FITC) or phycoerythrin (PE) (Becton & Dickinson, Mountain View, CA). The presence of τ/δ T cells was demonstrated by the monoclonal anti-TCR-τ/δ-1 (clone 11F2)(6) by an indirect fluorescence technique. Cells were stained by incubating 0.5 - 1 x 10^5 cells in 50 µl Hanks Balanced Salt Solution (Biochrom KG, Berlin) supplemented with 1 % bovine serum albumin and 0.1 % sodiumazide for 30 min at room temperature with 2 antibodies conjugated to different fluorochromes. After washing, cells were analysed on a FACScan flow-cytometer (Becton and Dickinson).

HLA typing

Spleen cells or peripheral blood mononuclear cells (obtained by Ficoll separation of heparinized blood) were typed for HLA class I antigens according to the standard NIH lymphocytotoxicity assay, and typed for HLA-DR by the two-colour fluorescence assay with a set of highly selected antisera (7).

Cell-mediated cytotoxicity assays

Biopsy-derived bulk cultures were tested for donor directed cytotoxicity in a standard 4-h ^{51}Cr release assay according to the European Standard Technique (8). As target cells we used donor derived cell lines and a panel of unrelated target cells (PHA T cell blasts or EBV transformed B cell lines) sharing one or more HLA antigens with the donor, and a third party control. The specificity for donor HLA class I (HLA A and B) or class II (HLA DR) antigens was determined by testing the cytotoxicity of the biopsy derived T

cell lines against a panel consisting of 5-10 (median 7) target cell lines. Each individual HLA-antigen was represented 1-4 times in the cell panels. Some representative cytotoxicity titrations are represented in figure 2. If the cytotoxicity against an HLA-antigen was difficult to interpret, like the example of HLA-B15 in figure 2 (left panel), this antigen was considered to be not tested. Target cells were incubated for 1 '1/2 hours at 37°C with 200 µCi ^{51}Cr (Na_2CrO_4 5 mCi/ml, specific activity 350-600 mCi/mg chromium, Amersham, UK). ^{51}Cr labelled target cells (2.5 x 10^3) were mixed with effector cells in 200 µl culture medium per well in 96 well U-bottom microtiter plates (Costar). Serial double dilutions with effector/target ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in 5% CO_2. Supernatants were harvested with a Skatron harvesting system (Skatron-AS, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA).

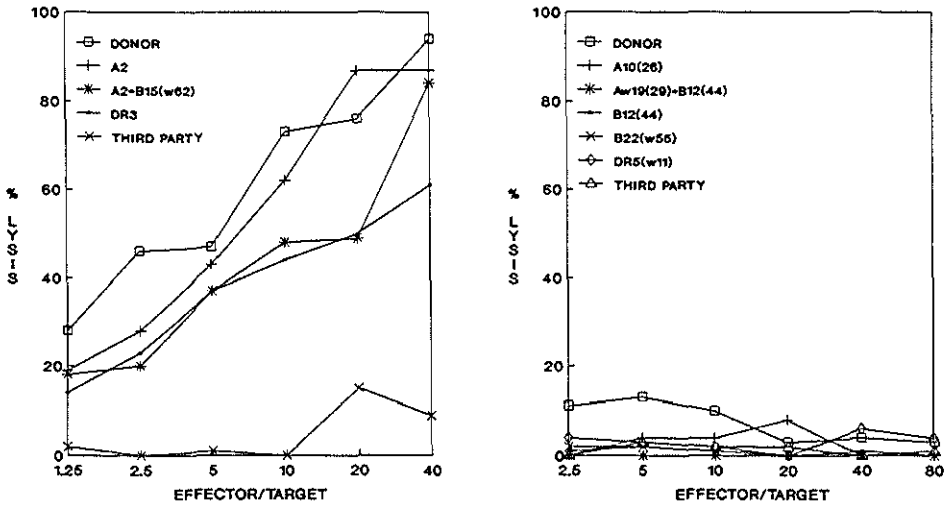

Figure 2 Representative example of a positive (left) and a negative cytotoxicity titration (right) of biopsy derived cultures from 2 different patients. The cultures were not restimulated with donor cells and were cultured for 40 and 26 days, respectively. In these examples, EBV transformed B cell lines were used as targets. The HLA-antigens shared by target cells and the heart donor are indicated. HLA-typing: left panel: Recipient: A1, A10(25), B18, B35, DR2(w15),DRw6(w13). Donor: A2, B15(w62), B18, DR2(w15), DR3. Right panel: Recipient: A2, A3, B35, B37, DR7, DRw10. Donor: A10(26), A19(29), B12(44), B22(55), DR5(11), DR7.

Spontaneous chromium release was determined by incubation of targets in 0.2 ml culture medium, and maximum release was obtained by adding 10% Triton X-100 detergent (5% v/v solution in 0.01 TRIS buffer) to the targets. Experimental release was measured, and specific lysis was calculated with the following equation:

$$\text{Specific lysis} = 100 \times \frac{\text{exp. release} - \text{spontaneous release}}{\text{max. release} - \text{spontaneous release}}$$

According to the recommendations of the European CML Workshop (8), cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector/target ratio of 20:1 or greater, and the slope of a graph was positive. Series of double dilution studies revealed that lysis percentages of autologous controls did not exceed 10%.

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CHAPTER 3

ALLOREACTIVE LYMPHOID INFILTRATES IN HUMAN HEART TRANSPLANTS[1]

Loss of class II directed cytotoxicity more than three months after transplantation

A.J. Ouwehand, L.M.B. Vaessen, C.C. Baan, N.H.P.M. Jutte, A.H.M.M. Balk, C.E. Essed, E. Bos, F.H.J. Claas and W. Weimar.

Abstract

From 535 endomyocardial biopsies (87 heart transplant recipients) 283 cell cultures could be generated. All cultures tested contained T lymphocytes and in most cases CD4 was the predominant phenotype at any time post transplant. A significantly higher proportion of CD8 dominated cultures was found among cultures from biopsies without histological signs of acute rejection (myocytolysis).

In the first three months post transplant 57% of cultures showed cytotoxicity against both class I and class II mismatched donor MHC antigens, compared with 33% at > 90 days. This proved to be due to a significant decrease in the number of cultures with HLA class II directed cytotoxicity.

This study shows that early after transplantation a heart transplant is infiltrated with activated donor specific cytotoxic T cells which recognize a broad spectrum of mismatched donor MHC antigens, and that in time this spectrum becomes more restricted.

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[1] Hum Immunol 1991;30:50.

Introduction

The diagnosis of rejection after clinical heart transplantation is based on histological criteria (1). Therefore endomyocardial biopsies (EMB) are taken at regular intervals after transplantation. This provides us with the opportunity to culture graft infiltrating cells, which makes it possible to evaluate growth patterns, phenotypic composition and function of these cells both in periods of graft stability and during rejection. The biopsies were cultured in interleukin-2 (IL-2) conditioned culture medium in the presence of irradiated autologous peripheral blood mononuclear cells (PBMC) as feeder cells, thereby assuming that only in vivo activated lymphocytes will proliferate (2,3). Neither donor nor third party cells were added within the first three weeks of culture to avoid in vitro activation. In the present report, we describe the phenotypic and functional characteristics of lymphocytes grown from EMB, and the relation of these findings with time after transplantation and histopathological diagnosis.

Methods

Patients

We studied 535 biopsies from 87 heart transplant recipients transplanted between January 1985 and February 1989. During this transversal study we received 2 to 18 EMB from each patient (median 4). EMB were taken 5 to 1587 days (median 114) after transplantation. All patients had received preoperative blood transfusions and all received cyclosporine (Sandimmune^{R}, Sandoz, Basel, Switzerland) and low dose prednisone as maintenance immunosuppression. Their actuarial graft survival at three years was 89%. Detection of acute rejection was performed by endomyocardial biopsy. Grading of the biopsies was according to Billinghams criteria of none, mild, moderate and severe rejection (1). For the diagnosis of moderate rejection, the coexistence of myocyte necrosis and mononuclear infitrates was required. Antirejection treatment was instituted in case of moderate rejection, and consisted of bolus steroids or, in case of ongoing rejection, with a two-week course of a polyclonal rabbit anti thymocyte globulin preparation. There were no cases of severe rejection. In the early posttransplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to once every 4 months at 1 year. After an acute rejection episode the next biopsy was taken 1 week following antirejection therapy. During right ventricular catheterisation four or five biopsy samples were obtained. Three or four were used for histologic evaluation, and one was placed in RPMI-1640 for tissue culture.

Culture method

Each biopsy was divided into 2 or more fragments and placed into 2 or more wells of a 96-well roundbottom tissue culture plate (Costar 3799, Cambridge, MA) with 200 µl culture medium in the presence of 10^5 irradiated (40 Gray) autologous PBMC as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% vol/vol lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. PBMC were isolated by Ficoll-Isopaque (δ = 1.077) density-gradient centrifugation. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Half the culture medium was refreshed every 2 to 3 days. When growth was observed the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing down or cell death was observed the cultures were restimulated by adding either 10^5 irradiated (40 Gray) donor spleen cells per well or, when available, 10^4 Epstein-Barr virus (EBV) transformed donor cells per well (irradiated with 80 Gray). This was not done in the first three weeks of culture.

B lymphoblastoid cell lines (B-LCL) originated from infection of fresh PBMC or spleen cells with EBV obtained from the marmoset cell line B95-8 as described by Moreau et al. (4). These cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, England).

T-lymphoblastoid cell lines were obtained by culturing PBMC or spleen cells in RPMI 1640 supplemented with 5% pooled human serum, 5% vol/vol Lymphocult-T (Biotest) and 1% phytohemagglutinin (PHA).

K562 was cultured in RPMI 1640 with 10% heat-inactivated foetal calf serum.

Phenotypic analysis

Surface differentiation antigens were analysed by two-color flow cytometry after staining with monoclonal antibodies directed against CD3 (anti-leu4) as a pan mature T cell marker, WT31 as a marker for the α/β chain of the T cell receptor (TCR), and CD8 (anti-leu2) and CD4 (anti-leu3) as T cell subset markers. CD16 (anti-leu 11) and CD56 (anti-leu 19) were used as markers for natural killer (NK) cells. Antibodies were directly conjugated to fluoresceine (FITC) or phycoerythrin (PE) (Becton & Dickinson, Mountain View, CA). The presence of τ/δ T cells was demonstrated by the monoclonal anti-TCR-τ/δ-1 (clone 11F2)(5) by an indirect fluorescence technique. Cells were stained by incubating 0.5 to 1 x 10^5 cells in 50 µl Hanks Balanced Salt Solution (Biochrom KG, Berlin) supplemented with 1 % bovine serum albumin and 0.1 % sodiumazide for 30 min at room temperature with 2 antibodies conjugated to different fluorochromes. After washing, cells were analysed on a fluorescence activated cell scan (FACScan) flow-cytometer (Becton & Dickinson).

Cell-mediated cytotoxicity

As described in chapter 2, a standard 4-h ^{51}Cr release assay was used to measure the cytotoxic capacity of the cultures against donor cells and a panel of unrelated target cells sharing one or more HLA antigens with the donor. Cytotoxicity was tested against donor cells and a panel of unrelated target cells sharing one or more HLA antigens with the donor, and a third party control. Fifty-nine bulk cultures could be tested before restimulation with allogeneic cells. The remaining cultures had to be restimulated in order to obtain sufficient numbers of cells. No effect of restimulation on cell-mediated lympholysis (CML) specificity could be demonstrated after repeated testing. Three types of target cells were used: PHA-blasts (HLA class I targets), B-LCL (class I and II targets) and the K562 cell line for assessment of NK cell activity. A standard 4-hour ^{51}Cr-release assay was performed (6) with one effector-target ratio of 20:1. When possible E:T ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in 5% CO_2. Supernatants were collected after 4 hours with a Skatron harvesting system (Skatron AS, Lier, Norway), and the release of ^{51}Cr was assayed in a Packard gamma-counter. Cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector:target ratio of 20:1 or greater, and the slope of a graph was positive (6). Series of double dilution studies revealed that lysis percentages of autologous controls did not exceed 10% (data not shown).

For statistical analysis of all data a X^2 test, with Yates correction when appropriate, was performed.

Results

Generation of lymphocyte cultures

A total of 283 lymphocyte cultures were established from 535 EMB (53%). From the majority of patients (72/87), cells could be successfully grown from at least one biopsy. From the remaining 15 patients only a few biopsies were available and these were almost always taken more than one year after transplantation. Cell growth was most successful from EMB taken within the second and third postoperative months (table 1). After three months a significant decrease of the growth percentage was observed ($p < 0.005$ when compared with all EMB taken in the first three months). Table 2 shows that the rate of establishing cultures is positively correlated with increasing histological rejection grade. Even when no mononuclear cells were detected by histology (grade 0 or 4) cell growth was obtained in 37% of the cases. These grade 0/4 biopsies showed the highest growth percentages between 11 and 180 days after transplantation with a peak of 64% in the second and third postoperative months and a decline to 33% after 180 days ($p < 0.01$ when the period between 11 and

180 days is compared with > 180 days). EMB showing histological signs of infiltrate (grades 1 and 2) yielded more cultures than grade 0/4 biopsies, independent of the time after transplantation. After the first 90 days a decline of the growth rate of grade 1 EMB was observed ($p < 0.01$). Grade 2 EMB always showed high growth percentages (table 2).

Table 1 Culture results and numbers of cultures analyzed for phenotype and cytotoxicity in relation to time after transplantation.

Days after transplantation	No. patients	No. EMB obtained	No. EMB growing (%)	Analyzes for phenotype	Tested in CML
0-10	30	31	7 (23)	6	4
11-30	31	80	46 (58)	39	24
31-90	32	125	91 (73)	74	49
91-180	34	96	55 (57)	36	25
181-365	32	83	31 (37)	21	7
> 365	50	120	53 (44)	24	17
Total	87	535	283 (53)	200	126

Table 2 Relationship of successful cellgrowth from EMB with histological rejection grades (Billingham's criteria) in different time intervals after transplantation.

	Grade 0, 4[1]		Grade 1		Grade 2	
Days posttransplant	No. EMB	growing (%)	No. EMB	growing (%)	No. EMB	growing (%)
0-10	26	3 (12)	5	4 (80)	0	0 (0)
11-30	25	10 (40)	46	30 (65)	9	6 (67)
31-90	25	16 (64)	83	60 (72)	17	15 (88)
91-180	26	14 (54)	59	32 (54)	11	9 (82)
181-365	42	9 (21)	41	22 (54)	0	0 (0)
> 365	75	29 (39)	44	23 (52)	1	1 (100)
Total EMB	219	81 (37)	278	171 (62)	38	31 (82)
No. patients	78	52	64	53	23	20

[1] Grades 0 and 4 EMB were grouped together because these show equal growth percentages.

Phenotypic analysis

Flow cytometric analysis of cell surface molecules of 200 cultures showed that the majority (89.5%) exclusively consisted of cells carrying the CD3 determinant. Almost all $CD3^+$ cells were $WT31^+$. The CD4/CD8 makeup of the T cells in 200 cultures is shown in table 3.

Table 3 Predominant phenotype of 200 cultures (from 59 patients) in relation to time after transplantation.

	Numbers of cultures						
	Mixed cultures				Single-cell-type cultures		
Days post transplant	> 60% CD4	CD4 = CD8	> 60% CD8	> 60% $CD4^-$ $CD8^-$	only CD4	only CD8	only $CD4^-$ $CD8^-$
0-30	18	13	8	0	3	3	0
31-90	25	15	19	0	12	3	0
91-180	10	5	6	1	11	3	0
> 180	17	5	9	2	3	6	3
Total EMB	70	38	42	3	29	15	3
No. patients	42	25	28	3	20	15	3

29 cultures contained only $CD4^+$ T cells and 15 consisted of $CD8^+$ cells only. In three cases exclusively $CD4^-CD8^-$ cells were found (TCR τ/δ^+ cells, described below). All except three recipients whose EMB cultures could be tested for phenotype had multiple mismatches for HLA class I and II with their donors. The cultures from one patient with only one HLA class II mismatch and no class I mismatches contained only $CD4^+$ cells (3 EMB). Five EMB from two other patients with only one HLA A or B mismatch and no HLA class II mismatches, yielded pure CD8 cultures as well as mixed cultures. One culture even contained $CD4^+$ T cells only. No significant difference in the number of mismatches between donor and recipient could be demonstrated between patients supplying pure CD8 or pure CD4 cultures (Mean number of mismatches: 2,80 vs 2,85 for HLA A, B and 1.60 vs 1.45 for HLA DR respectively). In the remaining 153 cultures both $CD4^+$ and $CD8^+$ cells were found, and the greatest fraction comprised cultures with CD4 as the predominant phenotype at any time after transplantation. In the cultures originating from biopsies with myocytolysis the preponderance of CD4 cells was more striking and a significantly lower proportion of CD8 dominated cultures was found (fig. 1, $p < 0.025$). In 16 cultures from 13 patients $CD3^+WT31^-11F2^+$ cells (TCR τ/δ^+) were found in amounts varying from 5 to 100% of the cultured cells (median 27%). The EMB were taken between 29 and 1324 days posttransplant (median 624 days). In four cases (four different

patients) the $\tau\delta^+$ cells dimly expressed CD8. The remaining cultures only contained $CD4^-CD8^-$ $\tau\delta^+$ cells. We found $\tau\delta^+$ cells significantly more often at more than one year after transplantation (11/24 vs 5/176 phenotyped cultures, $p < 0{,}001$). Three of four dimly $CD8^+$ $\tau\delta^+$ containing cultures originated from EMB taken more than 1 year posttransplant.
$CD3^-$ cells were found in 21 cultures (19 patients). In these cultures 7% to 96% of cells expressed CD16 and/or CD56 antigens (median 24%), and originated from EMB taken 6 to 1587 days posttransplant (median 148 days). In seven cultures from various patients the NK cells were dimly CD8 positive, six of them were grown from EMB taken in the first posttransplant year. We detected significantly more NK cells in cultures from biopsies taken more than one year after transplantation compared with the first post transplant year (8/24 vs 13/176, $p < 0.001$). These cells were never grown from biopsies with myocytolysis.

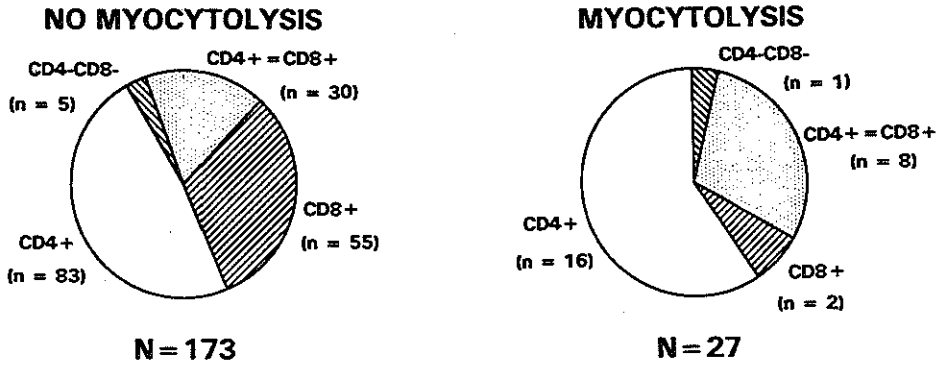

Figure 1 Proportion of cultures dominated by (> 60% of the cells in a culture) $CD4^+$, $CD8^+$ or $CD4^-8^-$ cells or containing equal amounts of $CD4^+$ and $CD8^+$ cells. The phenotypic composition of 173 cultures derived from EMB without myocytolysis (histology grade 0 and 1) was compared with that of 27 cultures from EMB with myocytolysis (grade 2). During acute rejection a decrease in the number of cultures containing predominantly $CD8^+$ cells was observed ($p < 0{,}025$).

Cell mediated cytotoxicity
From 126 EMB (47 patients) sufficient cells were generated to test cytotoxic functions (table 1). Kill of donor antigen-bearing target cells was found in 106 cultures. Table 4 shows the results of the specific CML reactivity. Donor specific cytotoxicity against HLA class I antigens (either alone or in

combination with reactivity against class II) was found in 75% of the cases, and against class II antigens (alone or in combination with class I) in 56%. Reactivity against both class I and class II antigens was detected in 48% of the bulk cultures and restricted reactivity (i.e. against either class I or class II antigens only) in 37%. Cultures originating from grades 1 and 2 EMB showed multispecific CML reactivity significantly more often when compared to grade 0 EMB cultures (table 4, $p < 0.01$), while most negative CMLs were found among grade 0 EMB cultures ($p < 0.005$, compared to grade 1 and 2 EMB cultures).

Table 4 CML reactivity of 126 bulk cultures (from 47 patients) against panel cells sharing either HLA class I or class II with the donor.

	CML specificity	grade 0,4	grade 1 and 2	total
< 90 days		n[1] = 18	n = 59	n = 77
		n (%)	n (%)	n (%)
	class I only	7 (39)	10 (17)	17 (22)
	class II only	1 (6)	5 (8)	6 (8)
	both class I and II	5 (28)	39 (66)	44 (57)
	negative	5 (28)	5 (8)	10 (13)
> 90 days		n = 16	n = 33	n = 49
		n (%)	n (%)	n (%)
	class I only	4 (25)	14 (42)	18 (57)
	class II only	2 (13)	3 (9)	5 (10)
	both class I and II	4 (25)	12 (36)	16 (33)
	negative	6 (38)	4 (12)	10 (13)
total		n = 34	n = 92	n = 126
		n (%)	n (%)	n (%)
	class I only	11 (32)	24 (26)	35 (28)
	class II only	3 (9)	8 (9)	11 (9)
	both class I and II	9 (26)	51 (55)	60 (48)
	negative	11 (32)	9 (10)	20 (16)

After 90 days posttransplantation a significant decrease in the number of cultures with reactivity against both HLA class I and II of the donor was found. This proved to be due to a decrease in the number of cultures with HLA class II directed cytotoxicity derived from grade 1 and 2 EMB (from 44/59 to 15/33 after 90 days, $p < 0.01$). Most negative CMLs were found among grade 0 EMB cultures ($p < 0.005$ compared to grade 1 and 2 EMB cultures).

[1] n=numbers of cultures.

In time a significant shift was observed from a predominantly multispecific to a more restricted CML specificity when the cultures from EMB taken before and after three months were compared (table 4). This proved to be due to a significant decrease in the number of cultures with HLA class II directed cytotoxicity derived from grade 1 and 2 EMB ($p < 0.01$). This decrease was not accompanied by a significant change in the phenotypic composition of the bulk cultures as CD4 remained the predominant phenotype at any time after transplantation, regardless of the CML specificity of these cultures. Similar phenotypic compositions were found in cultures with HLA class I directed cytotoxicity and in cultures without donor-directed CML reactivity.
Kill of the K562 cell line was observed in 26 of 107 tested bulk cultures. In 22 of these allospecific reactivity was also found. NK reactivity did not show a significant relation with time after transplantation or histological rejection grade. Only 7 of 26 cultures contained cells with the CD16 and/or CD56 phenotype of which two had 70% and 64% $CD8^{dim+}$ NK cells next to $CD4^{bright+}$ T cells. One of the latter cultures showed both donor specific killing and kill of K562, the other culture did not show donor specific lysis, but only killed K562. Five of eight tested $CD8^-$ NK cell containing cultures showed NK reactivity, two did not lyse donor antigen-bearing panel cells. All four tested cultures with high percentages of NK cells (>55%) lysed K562.

Rejectors versus nonrejectors
The group of 87 heart transplant recipients consisted of 59 patients who had experienced one or more rejection episodes (rejectors) and 28 patients who had never rejected their grafts (nonrejectors). There was no significant difference in the number of HLA mismatches between donor and recipient in both populations (mean number of mismatches 2.78 vs. 2.79 for HLA A,B and 1.41 vs 1.21 for HLA DR respectively).
Generation of lymphocyte cultures from EMB from both patient groups showed similar growth patterns in relation to time after transplantation and histological rejection grade (table 5). Cultures from biopsies from nonrejectors more often showed a predominance of $CD8^+$ T cells than EMB cultures from rejectors (table 6, $p < 0.025$). This was due to the significantly higher proportion of CD8 dominated cultures derived from histology grade 1 EMB in the nonrejectors ($p < 0.005$ compared with grade 1 EMB cultures from rejectors), especially in grade 1 EMB preceeding a grade 0 biopsy. No significant relation with time after transplantation could be demonstrated.
CML panel reactivity of EMB cultures from rejectors changed from a predominantly multispecific pattern in the first three months to a more restricted cytotoxicity thereafter (table 7). In the first three months 67% of cultures were cytotoxic against both HLA class I and class II, which decreased to 36% thereafter ($p < 0.01$). This was mainly due to a decrease of HLA class II-directed cytotoxicity. In the nonrejectors no significant decline in the fraction

of cultures showing multispecific cytotoxicity was found. In the first three postoperative months the number of cultures exhibiting cytotoxicity against both HLA class I and II was lower than in the rejectors. After three months no significant difference in cytotoxic specificities between rejectors and nonrejectors could be demonstrated.

Table 5 Relationship of successful cellgrowth from EMB with rejecotor or nonrejector status in different time intervals after transplantation.

	Rejectors			Nonrejectors		
Days posttransplant	No. patients	No. EMB	growing n (%)	No. patients	No. EMB	growing n (%)
0-10	17	17	5 (29)	13	14	2 (14)
11-30	18	44	25 (57)	13	36	21 (58)
31-90	20	72	53 (74)	12	53	38 (72)
91-180	24	73	42 (58)	10	23	13 (57)
181-365	23	59	25 (42)	9	24	6 (25)
> 365	36	87	41 (47)	14	33	12 (36)
Total	59	352	191 (54)	28	183	92 (50)

Table 6 Predominant phenotype of EMB cultures from 59 rejectors and 28 nonrejectors and relationship with histological rejection grade.

		Numbers of cultures				
	Histological rejection grade	> 60% CD4	CD4 = CD8	> 60% CD8	> 60% $CD4^- CD8^-$	total
Rejectors	0	13	3	13	3	32
	1	39	20	16	2	77
	2	16	8	2	1	27
		68	31	31	6	136
Non rejectors	0	14	1	4	0	19
	1	17	6	22	0	45
		31	7	26	0	64

Table 7 CML specificity of 87 bulk cultures from rejectors (33 patients) and 39 cultures from nonrejectors (14 patients).

	CML specificity	rejectors	non rejectors
< 90 days		n[1] = 48 (18 patients)	n = 29 (12 patients)
		n (%)	n (%)
	class I only	7 (15)	10 (34)
	class II only	2 (4)	4 (14)
	both class I and II	32 (67)	12 (41)
	negative	7 (15)	3 (10)
> 90 days		n = 39 (23 patients)	n = 10 (6 patients)
		n (%)	n (%)
	class I only	14 (36)	4 (40)
	class II only	5 (13)	0 (0)
	both class I and II	14 (36)	2 (20)
	negative	6 (15)	4 (40)

In the rejectors a significant decline in the number of cultures with multispecific reactivity was found (32/48 vs. 14/36 after 90 days, $p < 0.01$). This was mainly due to a decrease of HLA class II directed cytotoxicity (34/48 at < 90 days vs. 19/39 at > 90 days). In the nonrejectors this was less apparent.

[1] n: numbers of cultures.

Discussion

The present study shows that donor-specific cytotoxic lymphocytes can be grown from EMB taken at any time after transplantation, especially from grade 1 and 2 EMB. We also showed that CML panel reactivity changed in relation to time from a predominantly multispecific pattern to a more restricted one, which proved to be due to a significant decrease in the number of cultures with HLA class II-directed cytotoxicity. No corresponding change was observed in the phenotypic composition as CD4 remained the predominant phenotype in most cultures at any time after transplantation. This could mean that only a small fraction of the lymphocytes in the bulk cultures is responsible for the CML specificity, or that the responsible cytolytic T lymphocytes (CTL) have high affinity antigen receptors that do not require CD4 or CD8 molecules to stabilize antigen binding (7). MacDonald et al. showed that resistance of murine

CTL clones to inhibition with anti-CD8 monoclonal antibodies correlates with in vivo priming of these cells (8). Whether T cells carrying high affinity T cell receptors for antigen preferentially home in the allograft has not been investigated yet in the human. Our recent studies indicate that this may indeed be the case, especially during rejection (chapter 4).
Another finding in the present study was the higher incidence of NK cells and TCR τ/δ^+ cells among cultured graft infiltrating cells at one year after transplantation which, with one exception for the $\tau\delta^+$ cells, only originated from EMB without myocytolysis. The role of these cells in an allograft is not clear. They might play a role in maintaining graft stability or just be attracted to the site by lymphokines produced by MHC-restricted cells, macrophages or other types of cells. Whether $\tau\delta^+$ cells or NK cells play a role in the development of chronic rejection is still under investigation.
Part of the cultures dimly expressed CD8. These CD8 molecules did not seem to function as accessory molecules mediating class I-directed killing, for none of the bulk cultures containing high percentages of these cells showed donor-specific lysis. Some of the $\tau\delta^+$ cell-containing cultures have been cloned. Neither donor-specific cytotoxicity nor proliferation could be demonstrated in any of the $CD8^{dim+}$ or the $CD8^- \tau\delta^+$ clones (9). Furthermore, after culture of $CD8^+$ clones from one biopsy in recombinant IL-2 containing medium the CD8 expression disappeared (data not shown). The CD8 molecule might have been induced on the cells by the culture conditions in medium with a mixture of lymphokines, including IL-4. It has been demonstrated before that $CD8^{bright}$ can be induced on $CD4^+$ lymphocytes under the influence of IL-4, and that this molecule can be functional in class I mediated killing (10).
Several comparative studies of the CD4/CD8 profile of the T cells seen in situ (immunoperoxidase staining) and that of the lymphocyte cultures showed that the CD4/CD8 composition of lymphoid cells cultured from biopsies had a good correlation with the actual situation in the graft (11,12). The present study shows that most biopsies yield mixtures of $CD4^+$ and $CD8^+$ cells with CD4 as the predominant phenotype in most cases, which is in agreement with data of Fung et al. (13). In a limited number of observations others found CD8 as the predominant T cell subset in cultures from renal or cardiac transplant biopsies. This is thought to be due to the use of azathioprine in their immunosuppressive protocols (2, 12, 14).
We showed that a lower rejection grade was associated with a higher number of CD8-dominated cultures. Also in patients who never experienced acute rejections significantly more $CD8^+$ cells were found among infiltrating cells. These observations could suggest that these cells play a mitigating role in the rejection process. An alternative explanation for the greater predomination of $CD4^+$ cells during rejection might be that it is a consequence of higher HLA class II expression on graft tissue. We could not confirm the finding of Weber et al. (15) that cell growth from histologically negative EMB obtained in the

first postoperative month had a positive correlation with the cumulative incidence of subsequent histological rejection.
The lower incidence of HLA class II directed cytotoxicity after the first three months in the present study is in agreement with an extensive survey among renal transplant recipients (16). This survey showed that the effect of DR matching on the relative risk for graft failure was high in the first 5 months, while this effect had disappeared thereafter. On the other hand, the matching effect of HLA class I antigens was evident during the whole follow up period. In the present study we showed that class I-directed cytotoxicity remained relatively constant in time after transplantation. Several investigators have demonstrated the induction of MHC class I antigens on myocytes and increased expression of class I and II on interstitial structures during rejection (17-19), which makes the graft tissue more vulnerable to specific cell-mediated lysis (20). In some studies MHC expression in the allograft returned to normal after successful antirejection treatment (18), although others found persistence of expression of donor-type class II determinants on interstitial structures of the donor heart 1 and 2 years after transplantation (17, 21). It has been shown that, to a certain degree, HLA class II expressing dendritic cells of donor origin are replaced by recipient bone marrow derived cells (17, 22, 23). This may contribute to the lower incidence of donor-class II directed cytotoxicity. In vitro experiments have shown that lymphokines, particularly interferon τ, regulate the induction and upregulation of (donor) MHC expression on graft tissue and leucocyte binding and penetration through the endothelium (24,25), and that in the early posttransplant period, when the incidence of acute rejection is high, lymphokine producing cells are numerous in the graft (26). This is in agreement with our finding that the highest growth rates of alloactivated lymphocytes were found in the second and third months after transplantation. This peak of growth was observed in the rejector as well as nonrejector patient groups. Apparently the presence of activated lymphocytes in the graft does not always lead to allograft destruction. We found that cultures from nonrejector EMB taken in the first three postoperative months generally show a more restricted cytotoxicity than those from rejectors. This might indicate that in this period the number of alloreactive CTL clones in vivo is lower in nonrejectors, which may in turn have consequences for the development of myocyte injury.
Many speculations have been made about possible mechanisms involved in stabilisation of the graft and on the role different kinds of cells and lymphokines play in this process (27-30). Mechanisms that have been proposed to be involved are clonal deletion of antidonor alloreactive cells or specific suppression of alloreactive T cells. From earlier studies on circulating mononuclear cells CML hyporesponsiveness (27,28) and a reduction in the frequency of donor-reactive CTL precursors (29) have been reported in patients with well-functioning grafts. In contrast, another study on a patient with a well-functioning kidney graft showed that the frequency of donor-specific CTL-

precursors was still high, but these cells were not operational in vivo (30). We showed that biopsy-grown lymphocytes from allografts without acute rejection often still contain donor-directed cytotoxic cells. The mechanism that plays a major role in controlling the immune response in vivo is still unclear. Further investigations will address the question of whether specificity of graft infiltrating cells becomes more restricted because of diminished MHC class I and/or class II expression on donor heart tissue, or if it is caused by deletion or suppression of certain allospecific CTL clones, irrespective of the degree of allograft-MHC expression.

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CHAPTER 4

DETECTION OF CYTOTOXIC T CELLS WITH HIGH AFFINITY RECEPTORS FOR DONOR ANTIGENS IN THE TRANSPLANTED HEART AS PROGNOSTIC FACTOR FOR GRAFT REJECTION[1]

A.J. Ouwehand, C.C. Baan, D.L. Roelen, L.M.B. Vaessen, A.H.M.M. Balk, N.H.P.M. Jutte, E. Bos, F.H.J. Claas, W. Weimar.

Abstract

Alloreactive T lymphocytes are the initiators and effectors of acute rejection of organ transplants, and T cells with high affinity receptors for antigen might especially be implicated in this process. It has been shown that the cytotoxic capacity of cytotoxic T lymphocytes (CTL) with low-avidity for alloantigens can be inhibited with CD8 monoclonal antibodies (MAb), while high-avidity CTL are not affected. To investigate whether the presence of such high-avidity cells in human heart transplants may be predictive for acute rejection, we analyzed their frequency in cultures derived from endomyocardial biopsies in 19 patients, 9 of whom had never experienced acute rejection and 10 who had had one or more rejection episodes. In the rejectors, already before histological signs of rejection (myocyte damage) had developed, significantly higher donor-reactive CTL frequencies were found compared with the non-rejectors (medians of 10.586 versus 1.169 reactive cells per 10^6 tested cells, $p = 0.002$). After CD8 inhibition, the difference between rejectors and non-rejectors was even more pronounced ($p < 0.001$).

[1] Transplantation 1993;56:1223.

In patients with rejection, the number of CD8 resistant, high-avidity CTL was higher than 1000 per million cells in all cases, while in patients who had never experienced rejection this number was less than 1000. As these CTL characteristics are already present before the first histological signs of rejection have developed, this might be used as a prognostic factor.

Introduction

Acute rejection of organ transplants is mediated by antigen-specific T lymphocytes directed against products of the Major Histocompatibility Complex and other transplantation antigens (1-3). However, even in patients who will never clinically reject their grafts, these cells may be abundantly present, and can be propagated from their graft tissue as easily as from tissue specimens from organs that will be rejected (4,5). This implies that there is a functional difference between these infiltrating cells, for instance in avidity for donor HLA antigens. The cytotoxic function of T lymphocytes with high-affinity T cell receptors for antigen was shown to be resistant to inhibition with CD8 monoclonal antibodies in vitro, indicating that these cells apparently do not need the CD8 molecule to stabilize antigen binding (6). On the other hand, T cells with low-affinity T cell receptors could easily be inhibited.
To investigate whether the presence of high-avidity donor-reactive cytotoxic T lymphocytes (CTL) in human transplants is relevant for the acute rejection process, we analyzed CTL frequencies in endomyocardial-biopsy derived cells of 19 heart transplant recipients, 9 of whom had never experienced acute rejection and 10 who had had one or more rejection episodes.
We demonstrate here that there are marked differences between the characteristics of cytotoxic T cells infiltrating the grafts of patients with and without acute rejections, and that these differences can already be found early after transplantation, before the first histological signs of rejection have developed.

Methods

Patients
We studied 19 heart transplant recipients transplanted between April 1988 and October 1990, 10 of whom had experienced one or more acute rejection episodes during the first posttransplant year (1-5, median 3), and 9 patients who had never experienced rejection (nonrejectors). All patients had received preoperative blood transfusions and received only cyclosporine

(Sandimmune^R, Sandoz, Basel, Switzerland) and low dose prednisone as maintenance immunosuppression.
The mean number of HLA mismatches between donor and recipient for the A, B, and DR antigens was 1.26, 1.63 and 1.32, respectively, and was comparable in patients with and without rejection. Before transplantation, none of the patients had HLA alloantibodies as measured in the standard National Institute of Health complement-dependent cytotoxicity assay.
Detection of acute rejection was performed by histological examination of endomyocardial biopsies (7). During each of the 15 biopsy procedures in the first posttransplant year, four or five fragments of endomyocardium were obtained. Three or four were used for histologic evaluation, and one was placed in interleukin-2 conditioned culture medium for cell culture.
For the diagnosis of clinically relevant rejection, the coexistence of myocyte necrosis and mononuclear infiltrates was required. In these cases, antirejection treatment was instituted, which consisted of a 2-week course of rabbit antithymocyte globulin in case of rejection within the first month (9 patients), and a 3 day course of methylprednisolone intravenously in a dose of 1 gram daily in later rejections (8 patients). Four patients received an additional course of OKT3 for ongoing rejection.
From each of the patients serially cultured biopsies were available (4). For the present study, we selected one of these biopsy-derived cultures from each patient on the basis of the following criteria: they had to be taken before antirejection therapy was instituted (non rejectors: from a comparable period), and should contain cytotoxic $CD8^+$ cells that were directed against donor HLA class I antigens. The thus selected cultures had been derived from biopsies that were taken 28 days after transplantation (median, range: 14-169 days). The median culture period was 34 days (range 21-91 days). In the rejector group, 6 of the biopsies were taken 5-35 days (median 17) before a histologically proved, clinically relevant acute rejection episode and 5 other biopsies were taken during rejection.

Lymphocyte cultures were established from endomyocardial biopsies as described previously (4). In brief, each biopsy was cultured in a 96 well round bottom tissue culture plate (Costar, Cambridge, MA) with 200 µl culture medium per well, in the presence of 10^5 irradiated (30 Gy) autologous peripheral blood mononuclear cells as feeders. The plates were cultured in a humidified atmosphere at 37°C in 5% CO_2. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland), 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, supplemented with 10% pooled human serum and 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of interleukin-2.

Allogeneic target cells
Phytohaemagglutinin-blasts were obtained by culturing donor spleen cells for at least 5 days in the presence of 1% PHA-M (Difco, Detroit, MI) in culture medium supplemented with 5% pooled human serum and 5% lymphocult-T (Biotest). EBV transformed B cell lines were derived as described previously (8).

Phenotypic analysis
The cultures were analyzed for CD4 and CD8 expression by two-color flow cytometry after staining with the monoclonal antibodies anti-leu3 and anti-leu2 (Becton Dickinson, Mountain View, CA). The antibodies were directly conjugated to fluorescein or phycoerythrin. Cells were analyzed on a fluorescence-activated cell flow-cytometer (FACScan, Becton Dickinson).

Cell-mediated cytotoxicity assays
Biopsy-derived bulk cultures were screened for donor-directed cytotoxicity in a standard 4-h ^{51}Cr release assay according to the European Standard Technique (9). ^{51}Cr labelled target cells (2.5×10^3) were mixed with effector cells in 200 µl culture medium (containing 10% pooled human serum) per well in 96-well U-bottom microtiter plates (Costar). As target cells we used donor derived cell lines and a panel of unrelated target cells (phytohaemagglutinin T cell blasts or EBV-transformed B cell lines) sharing one or more HLA antigens with the donor, and a third party control. The specificity for donor HLA class I (HLA A and B) or class II (HLA DR) antigens was determined by testing the cytotoxicity of the biopsy-derived T cell lines against a panel consisting of 5-10 (median 7) target cell lines. Serial double dilutions with effector:target ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were harvested using a Skatron harvesting system (Skatron-AS, Norway), and the release of ^{51}Cr was assayed in a Packard gamma counter. According to the recommendations of the European CML Workshop (9), cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector:target ratio of 20:1 or greater, and the slope of a graph was positive. Series of double dilution studies showed that lysis percentages of autologous control cell lines did not exceed 10%.

Mixed lymphocyte reaction (MLR)
Peripheral blood mononuclear cells from 10 patients (5 with and 5 without rejection episodes) were isolated from blood samples obtained on the same day as the biopsy. Two million peripheral blood cells per well were cultured for 7 days in 24-well plates (Costar) with 2 million irradiated (30 Gy) donor spleen cells, or other allogeneic cells that shared HLA antigens with the donor. The culture medium consisted of RPMI-1640 (Dutch Modification)

with the above-mentioned additions, supplemented with 10% pooled male human serum.

Limiting dilution analysis

Limiting dilution cultures were set up in 96-well round-bottom microculture plates (Costar). Graded numbers of responder cells were cultured in 24 replicates with 50,000 irradiated (30 Gy) stimulator cells in a total volume of 0.2 ml. MLR cells were titrated in 8-fold double dilutions starting from 25,000 to 100,000 cells per well, and endomyocardial biopsy derived cells from 10,000 to 15,000 cells per well, depending upon the number of cells available. In 4 patients, lower cell numbers could be tested, with the limiting dilution starting from 1000-5000 biopsy derived cells per well. As stimulator cells we used donor spleen cells or, when not available, other allogeneic spleen cells that shared HLA antigens with the donor, and a third party control. The culture medium was supplemented with 10% pooled male human serum and 20 U/ml of recombinant human interleukin-2 (Biotest). Limiting dilution microcultures were incubated for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. At day 7 the microcultures were split in 2, and each well was individually tested for cytolytic activity against 2.5 x 10^3 ^{51}Cr labelled HLA-class I specific target cells (phytohaemagglutinin blasts of donor origin or third party cells). Half of the split wells was tested in the presence of CD8 monoclonal antibodies, as described below. The other half of the wells was tested against target cells only. Maximum and spontaneous release were determined in 5-fold. Microcultures were considered cytolytic when the experimental lysis percentage exceeded 10%.

CD8 inhibition studies

The CD8 monoclonal antibody FK18 (a mouse-anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule; a gift of dr. F. Koning, Dept. of Immunohaematology, Academic Hospital Leiden, The Netherlands) (10,11) was used as a 1:300 dilution of ascitic fluid. Before addition of the targets, FK18 was added to each well, mixed with effector cells and preincubated during 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2. Figure 1 shows a dose-response curve of FK18 inhibition of the cytolytic antidonor response of an endomyocardial biopsy-derived culture. In all dilutions used, a significant inhibition of donor specific lysis was found. As a control antibody, RIV6 (a mouse anti-human antibody of the IgG1 subclass, which is directed against the CD4 molecule) was used as a 1:300 dilution of ascitic fluid.

Statistical analysis

Frequencies of cytotoxic T lymphocytes (expressed as the number of cytotoxic cells per 10^6 cells) and their 95% confidence intervals were

calculated by the jackknife procedure for maximum likelihood (12,13). The calculated frequencies were accepted when the goodness-of-fit did not exceed 12. The significance of differences between the groups of patients was analysed with the Mann-Whitney test.

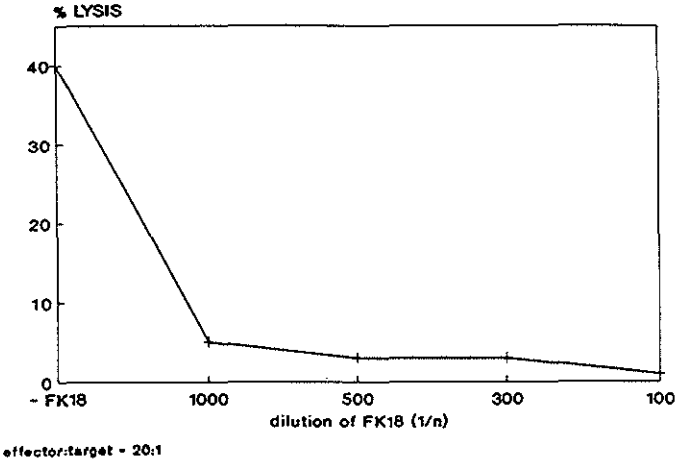

Figure 1 Dose response curve of CD8 inhibition of the donor-directed cytotoxic response of a CD8 sensitive endomyocardial biopsy derived T lymphocyte culture. This curve shows that the monoclonal antibody used (FK18) is very effective in the dilution used in our experiments (1:300).

Table 1 Frequencies of CTL in endomyocardial biopsy derived lymphocyte cultures reactive against donor and third party cells.

CTL FREQUENCY ESTIMATES (CTL/million cells)	rejectors (10 patients)	non rejectors (9 patients)
against donor cells	4,026 - > 60,795	< 28 - > 48,810
median	10,586*	1,169
third party cells	< 3 - 5,312	< 2 - 1,444
median	< 8	< 3
after CD8 inhibition	rejectors (10 patients)	non rejectors (9 patients)
against donor cells	1,845 - > 60,795	0 - 871
median	6,415†	119
third party cells	0 - 29	0 - 358
median	0	0

From 10 heart transplant recipients with and 9 without acute rejection episodes one biopsy derived culture was analyzed.

* $p = 0.002$ when compared with nonrejectors (Mann-Whitney test).

† $p < 0.001$ when compared with nonrejectors (Mann-Whitney test).

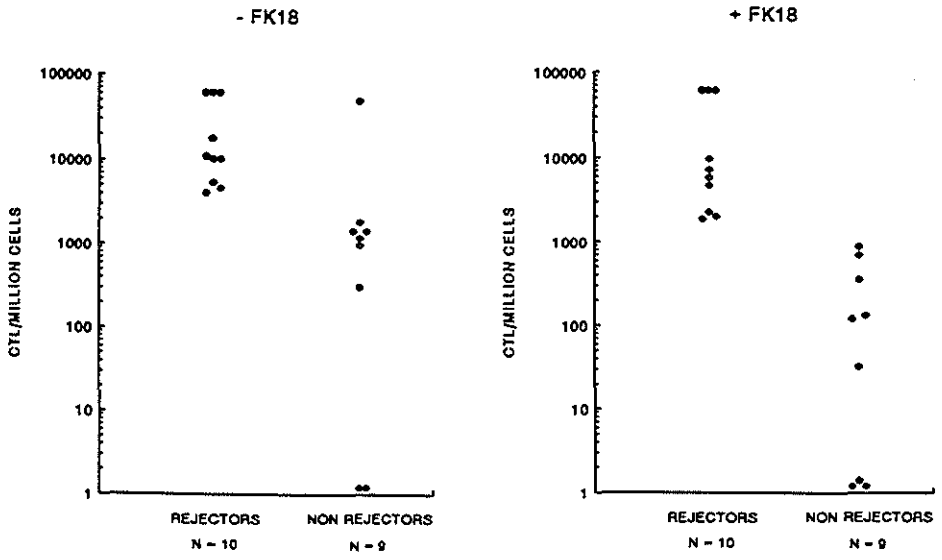

Figure 2 Frequencies of donor-reactive cytotoxic T lymphocytes in biopsy derived cultures from patients with and without rejection as measured in limiting dilution experiments. A. CTL frequencies against donor cells in the absence of CD8 monoclonal antibody. In the rejectors significantly higher frequencies were measured ($p = 0.002$, Mann-Whitney test). B. CTL frequencies after incubation with CD8 monoclonal antibodies. A greater difference between rejectors and nonrejectors was found ($p < 0.001$).

Results

Sensitivity of alloreactive CTL frequencies to inhibition with CD8 monoclonal antibodies, and the relation with rejector or non-rejector status

The frequencies of alloactivated graft infiltrating cytotoxic T cells were determined in endomyocardial biopsy-derived T cell lines from 19 patients, using a limiting dilution assay. All tested cultures had been expanded without the presence of donor cells. In the absence of CD8 antibodies, the frequencies of cytotoxic T lymphocytes that were reactive against donor cells varied from 0 to > 60,795 per million tested cells (median 9,547). In the rejectors, the frequencies were significantly higher compared with the nonrejectors (table 1, figure 2A, $p = 0.002$). In 2 nonrejectors no frequency was measurable. After CD8 inhibition, the difference between rejectors and nonrejectors was even more pronounced, due to a greater sensitivity to CD8 in the nonrejector group: we found median frequencies of 6,415 (rejectors) and 119 (nonrejectors) CD8-resistant donorreactive CTL/million (figure 2B,

$p < 0.001$). Incubation with CD8 monoclonal antibody resulted in a median inhibition of 13% in the rejectors, and 75% in the nonrejectors. In patients with rejection the number of CD8-resistant, high-avidity CTL was higher than 1,000 per million cells in all cases, while in patients who had never experienced rejection this number was less than 1,000.
In biopsies taken during rejection, the highest frequencies of CD8 resistant CTL were found (median: 60,795 per 10^6 cells, table 2). But even in prerejection biopsies a significantly higher number of donor-reactive CTL was resistant to CD8 inhibition when compared with biopsies with the same histological classification in the nonrejector group (medians of 5,196 and 119 per 10^6 cells, respectively, table 2). These biopsies were taken 5-35 days (median 17) before an acute rejection episode.

Table 2 Frequencies of CD8 resistant, high-avidity donor-reactive cytotoxic T lymhocytes in biopsy derived T cell lines from patients with and without rejection: relation with histological rejection grade*.

	non rejectors	rejectors	
	biopsy without myocyte necrosis* (n = 9)	biopsy without myocyte necrosis* (n = 6)†	biopsy with myocyte necrosis* (n = 5)
Frequency CD8 resistant cytotoxic T cells, range	0 - 871	1,978 - 9,547	1,845 - > 60,795
median	119	5,196	60,795
Fraction of CD8 resistant cells (median %)‡	25%	78%	100%

Frequencies are expressed as number of reactive cells per million tested cells. Even when only biopsies without histological signs of rejection were considered, significantly higher frequencies were found in the rejector group ($p < 0.001$, Mann-Whitney test).
* Histological rejection grade according to the criteria of Billingham[7].
† These biopsies were taken 5-35 days (median 17) before an acute rejection episode.
‡ Fraction of CD8 resistant, high avidity CTL within the total donor-reactive cytotoxic T cell pool.

Flow cytofluorometric analysis revealed that the $CD8^+$ cells in both inhibited and uninhibited cultures had a comparably bright expression of the CD8 antigen, as is usually seen in T cell populations. Endomyocardial biopsy-

derived T cell cultures contained 22-79% (median 56%) $CD8^+$ cells in the rejector group, and 17-88% (median 48%) in the nonrejectors.
From one patient with rejection, cultures from 2 serial biopsies were tested in the same limiting dilution assay. In both cases, comparably high lysis percentages of donor cells were found at all dilutions. Figure 3 shows the cytotoxicity titration of cells derived from one of the biopsies that was taken 6 days before the first acute rejection episode. Incubation with CD8 monoclonal antibodies gave no inhibition of the donor-specific cytolysis. The frequencies against third party cells were low in both cases (50 and 386 cytotoxic cells per million, respectively).
Figure 4 shows an example of a limiting dilution experiment on biopsy-derived cells from a non-rejector. The cytotoxic T cell frequency against donor cells was fully inhibited by the CD8 monoclonal antibody.

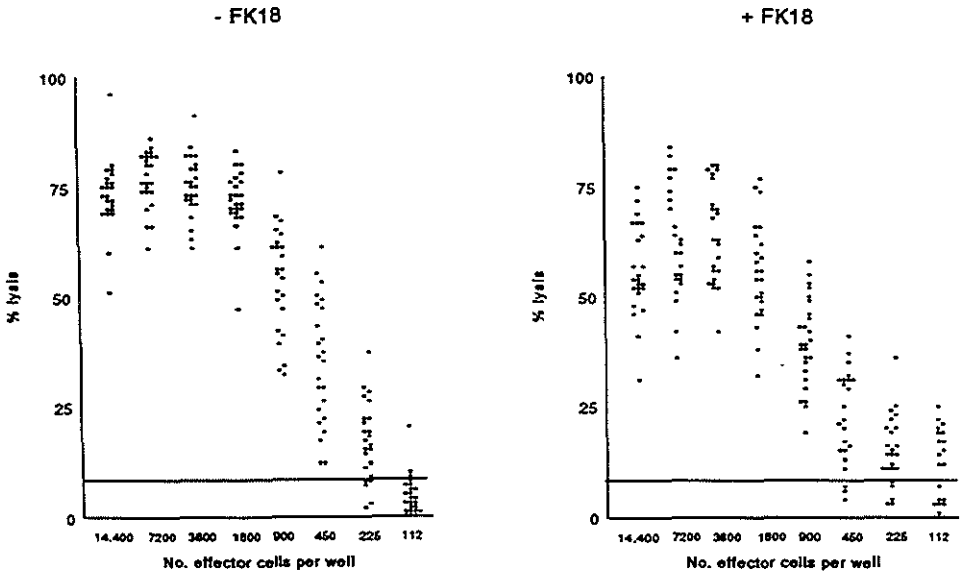

Figure 3 Limiting dilution experiment of a culture derived from a biopsy taken at day 23 after transplantation, 6 days before histological signs of rejection were evident. The donor-reactive CTL frequency estimate was 4,577 per million cells (2A)(95% confidence limits: 3,350-5,804). After CD8 inhibition (2B), a frequency of 5,740 per million was found (95% confidence limits: 3,852-7,628). The frequency of third party-reactive T cells in this specific case was 50 per million cells (95% confidence limits: 13-87), and 21 per million after CD8 inhibition.

The next biopsy, taken from the same patient at 29 days after transplantation during a rejection episode, yielded 3,057 donor reactive T cells per million cells (95% confidence limits: 2,155-3,960), all of which were again resistant to CD8 inhibition (95% confidence limits: 1,845-3,190). Both cultures were tested in the same limiting dilution experiment.

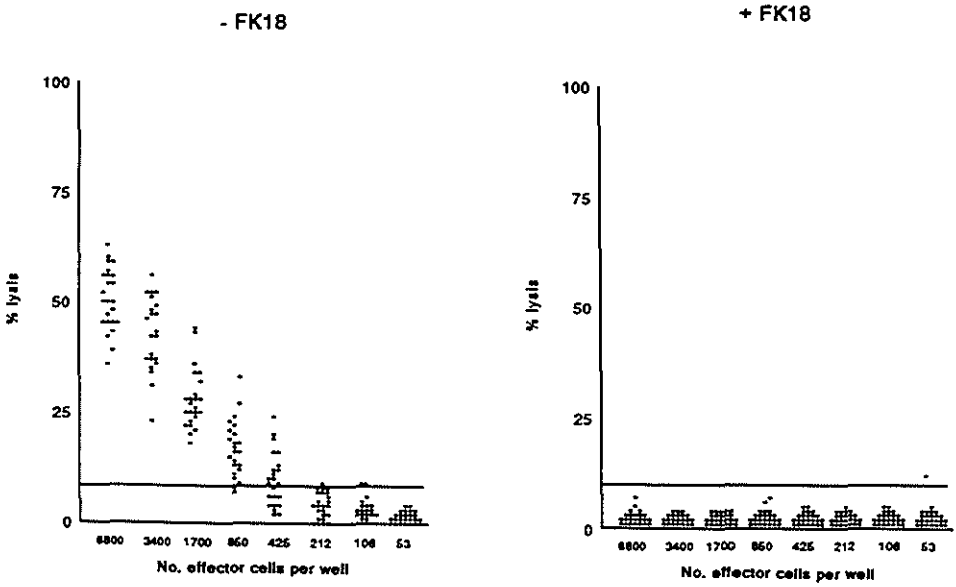

Figure 4 Limiting dilution experiment of a culture derived from a biopsy taken 20 days after transplantation from a patient without rejection. A. The donor-reactive CTL frequency estimate was 1,444 per million cells (95% confidence limits: 1,090-1,798), and was fully inhibited by the CD8 monoclonal antibody (3B).

Control experiments

The frequencies of cytotoxic T cells against third party controls were unmeasurable (in 5 rejectors and 5 nonrejectors) or, when measurable, were lower than those against donor cells, except for one obtained from a patient without rejection. All third-party reactive cells were strongly sensitive to CD8 inhibition: a median inhibition of 92% in the rejector group and 90% in the nonrejector group was found.

Three of the CD8-resistant endomyocardial biopsy-derived T cell lines were also tested against donor and third party cells in the presence of an antibody directed against CD4. In these experiments, neither CD4 nor CD8 could inhibit donor directed cytotoxicity.

To analyze whether the above-described characteristics of infiltrating cells derived from the grafts of rejecting or nonrejecting patients could also be found in the peripheral blood, we tested cells derived from blood samples that were obtained simultaneously with the biopsies in 10 patients (5 rejectors and 5 nonrejectors). Before subjecting them to the limiting dilution assay, the cells were stimulated with donor cells in a mixed lymphocyte reaction. In the thus-generated alloreactive T cell lines, the frequencies of donor reactive cytotoxic cells were comparable in rejectors and nonrejectors, and varied from 51 to 30,397 per million cells (median 1,348). Incubation with CD8 monoclonal antibody resulted in a median inhibition of 79% and 66%, respectively (not significant). This inhibition percentage was in the same order as that found for biopsy-derived T cell lines in patients without rejection. The measured frequencies are shown in table 3. Both in rejecting and nonrejecting patients, relatively high frequencies of third-party reactive CTL were detected among the peripheral blood-derived T cell lines (51 - 6,133 per million, table 3). These could be inhibited by the CD8 monoclonal antibody in all cases (a median inhibition of 83%).

Table 3 Frequencies of CTL in peripheral blood-derived lymphocyte cultures reactive against donor and third party cells.

CTL FREQUENCY ESTIMATES (CTL/million cells)	rejectors (5 patients)	non rejectors (5 patients)
against donor cells	51 - 30,397	74 - 13,588
median	1,185	874
third party cells	51 - 2,478	80 - 6,133
median	483	449
after CD8 inhibition	rejectors (5 patients)	non rejectors (5 patients)
against donor cells	< 1 - 6,490	27 - >6,133
median	946	293
third party cells	0 -1,520	0 - 1,269
median	70	89

From each patient, one peripheral blood-derived lymphocyte culture was analyzed. Blood samples were taken simultaneously with the endomyocardial biopsies. Before the limiting dilution assay, the cells were stimulated with donor cells in a mixed lymphocyte reaction.
The frequencies of donor- and third party reactive CTL were comparable in rejectors and nonrejectors (no statistically significant differences), and were all strongly sensitive to CD8 inhibition.

Our findings show that there is a marked difference between the number of donor-reactive cytotoxic T cells infiltrating the transplanted heart of patients who will reject their grafts and those who will never experience acute graft rejection. Both the total donor-reactive cytotoxic T cell pool and, even more evidently, the frequencies of CD8-resistant donor-reactive cytotoxic T cells, were found to be significantly higher in heart transplant recipients with rejection. This phenomenon was already present before histological signs of rejection (myocyte damage) had developed.

Previous experimental and clinical data (4, 14-16) have shown that both in individuals who tolerate their grafts and in those who will reject their grafts, cellular infiltrates are present that contain donor-reactive cells that have cytolytic potential in vitro. However, not in all graft recipients is this function exerted in vivo. Previously, we also showed that the total yield of cytotoxic cultures that could be derived from endomyocardial biopsies was comparable in both patients with and without rejection in the first 3 months after transplantation, but we found a different reactivity pattern: the T cell cultures from rejecting heart transplant recipients generally were cytotoxic against a broader range of mismatched HLA antigens when compared with those derived from the hearts of nonrejecting patients (4). This pointed to the presence of a more polyclonal infiltrate in the grafts of rejecting patients.

Orosz and coworkers (17,18) demonstrated that rejecting sponge allografts in mice acquire 3 types of cytotoxic lymphocytes: donor-reactive precursor cells, donor-reactive maturated (in vivo primed) cells and precursor cells with irrelevant specificity. In the present study, evidence for such a heterogeneous composition of the infiltrating cells was found in the transplanted heart. The biopsy derived T cell lines consisted of mixtures of CD8 resistant and CD8 susceptible donor-reactive CTL and variable, but generally low frequencies of third-party reactive cells that were CD8 susceptible.

It has been demonstrated that during and before a histologically proved rejection, alloactivated cytotoxic T lymphocytes preferentially accumulate in the graft (17-20). During cytotoxic T cell responses in vivo, probably a selection takes place for T cells of high affinity at sites where high concentrations of the specific antigen are present (6,17,18). This preferential homing and expansion of mature alloreactive cells in the graft may be the consequence of increased lymphokine production by cells invading the transplant (21), and by increased expression of transplantation antigens and adhesion molecules on graft tissue, factors that also mutually influence each other (1,2,22). There is increasing evidence that particularly CTL clones with high-affinity T cell receptors for antigen do not need CD8 molecules to stabilize antigen binding, contrary to cells with low-affinity receptors (6, 23-

26). We found that in biopsies taken during rejection, the biopsy-derived T cell lines mainly consisted of these CD8 resistant, high-affinity donor-reactive CTL. Furthermore, even in prerejection biopsies elevated fractions of these cells were found when compared with biopsies from the grafts of nonrejecting patients with the same histological classification in the same period after transplantation. Apparently a high frequency of CD8 resistant, high-avidity donor-reactive CTL (more than 1000 per million cells) is a prerequisite for tissue damage, and could predict whether a patient is a high responder to foreign HLA antigens, and thus is liable to develop acute rejection.

The lack of association in our patient group between graft rejection and frequencies of CD8 resistant donor-reactive CTL in the peripheral blood illustrates that the graft is the most informative site for the detection of cells that are relevant for rejection, a finding that is consistent with the observations of others (17-20).

No evidence of humoral sensitization against foreign HLA antigens caused by blood transfusions or pregnancies was observed in any of the patients before transplantation. Apparently some patients had become immunized against transplantation antigens present on the transplanted donor heart, to such a degree that high-avidity donor-reactive cytotoxic T cells were able to accumulate and expand in the graft, in spite of adequate cyclosporine treatment.

Others have shown that there is individual variability in sensitivity to cyclosporine of alloreactive T cells derived from the peripheral blood (27-29) and from transplanted organs (30,31). Furthermore, previously activated cytotoxic T cells were found to be least sensitive to cyclosporine (27,32). Our previous studies (11) in highly sensitized patients who were waiting for a renal transplant showed that the reactivity of alloactivated cytotoxic T cells directed against unacceptable mismatches (HLA antigens against which the patient had formed allo-antibodies) could not be inhibited with cyclosporine. In the same patients, the alloreactive cells were also refractory to inhibition with CD8 antibodies, indicating that these in vivo-maturated cells had high affinity receptors for their specific antigen.

From the present study it is clear that limiting dilution analysis in the presence of CD8 antibodies can be used to discriminate between those graft-invading donor-reactive CTL that are harmless to an allograft and those that are most likely to cause myocyte necrosis. The fact that the highest frequencies of donor-reactive CTL with high affinity T cell receptors were found in the grafts of patients with rejection indicates that these cells are indeed the effectors of acute graft rejection. Detection of high frequencies of these cells may identify those patients who are high responders to certain foreign HLA antigens, and who are at risk of developing one or more acute rejection episodes.

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CHAPTER 5

CHARACTERISTICS OF GRAFT INFILTRATING LYMPHOCYTES AFTER HUMAN HEART TRANSPLANTATION: HLA MISMATCHES AND THE CELLULAR IMMUNE RESPONSE WITHIN THE TRANSPLANTED HEART[1]

A.J. Ouwehand, C.C. Baan, L.M.B.Vaessen, N.H.P.M. Jutte, A.H.M.M. Balk, E. Bos, F.H.J.Claas and W. Weimar.

Abstract

The influence of HLA mismatches between donor and recipient on the phenotypes, function and specificity of T lymphocyte cultures derived from endomyocardial biopsies was studied in 118 heart transplant recipients. In case of HLA-DR mismatches, the majority of the endomyocardial biopsy (EMB) derived cultures were dominated by CD4+ T cells, while in patients with HLA-A and B mismatches but without DR mismatches $CD8^+$ T cells comprised the predominant T cell subset. Cytotoxicity against donor antigens was observed in 75% of the cultures. A significantly ($p < 0.005$) lower proportion of the cultures showed cytotoxicity against HLA-A antigens (36%) when compared to HLA-B (53%) or HLA-DR (49%). An HLA-A2 mismatch elicited a cytotoxic response that was comparable to that found against HLA B and DR antigens: 62% of the cultures from HLA-A2 mismatched donor-recipient combinations was reactive against A2. A higher

[1] Hum Immunol, in press.

number of A, B or DR mismatches resulted in a higher number of cytotoxic cultures directed against these antigens. A higher number of HLA-B and DR mismatches was associated with a lower freedom from rejection. Our data indicate that, despite the use of adequate immunosuppressive therapy, the degree of HLA matching plays a crucial role in the immune response against a transplanted heart, resulting in a significant effect on freedom from rejection.

Introduction

Products of the major histocompatibility complex play a major role in the immune response against a transplanted organ [1,2]. In extensive studies in kidney transplant recipients a positive effect of HLA matching on graft survival was reported, especially for HLA-B and DR antigens [3-8]. The beneficial effect of DR matching was found to be most evident in the first post operative months, while the effect of matching for HLA-B antigens lasted longer. These studies were based on the assumption that all of the HLA-alleles had the same antigenic weight. Busson and coworkers [9] showed in kidney transplant recipients with only one incompatible HLA-antigen, that some of the HLA-A antigens were associated with a lower transplant survival than others, while at the B locus, there was no significant difference in survival rate among the different antigens.

The importance of HLA matching for heart allograft survival is still debated, mainly because of the limited numbers of patients studied and, more importantly, the low numbers of well matched grafts performed, as donor hearts are randomly allocated without reference to HLA matching. Nevertheless, a beneficial effect of HLA matching has been found for cardiac graft survival [10,11], the incidence of steroid resistant rejection [12] or the freedom from rejection of the transplanted heart [13].

Acute allograft rejection is mediated by immunocompetent lymphocytes of the graft recipient that interact with allogeneic determinants expressed on the grafted organ. Recognition of both HLA-class I and class II allogeneic differences by both helper and cytotoxic T lymphocytes precipitates a cascade of reactions that results in a cytotoxic response directed against cells bearing these antigens, and thus in parenchymal damage of the graft tissue. [14-20]. In previous studies [21-25] we and others showed that graft infiltrating lymphocytes can be cultured from cardiac graft tissue specimens. During acute rejection episodes, a higher proportion of these biopsies yielded lymphocyte cultures, of which the majority was cytotoxic against donor derived cells.

The influence of HLA mismatches between donor and recipient on phenotypes and effector function of graft infiltrating cells has never been

systematically studied. Therefore we analyzed the effect of HLA-A, B and DR mismatches on the functional and phenotypic characteristics of these cells in a large series of endomyocardial biopsies from 118 heart transplant recipients. Moreover, we investigated the immunogenicity of individual mismatched HLA-antigens.

Methods

Patients

We studied endomyocardial biopsy derived graft infiltrating cells from 118 heart transplant recipients transplanted between February 1988 and January 1990. All patients had received preoperative blood transfusions and all received cyclosporine (Sandimmune^{R}, Sandoz, Basel, Switzerland) and low dose prednisone as maintenance immunosuppression. The actuarial patient survival was 89% at 4 years. The mean number of mismatches between donor and recipient was 1.25, 1.62 and 1.40 for HLA-A, B, and DR, respectively. Table 1 shows the distribution of the patients among the different match groups. HLA histocompatibility was based on matching for broad specificities. Homozygosities were considered as one mismatched antigen.

Table 1 Distribution of the 118 patients among the different mismatch groups.

	Numbers of patients		
	0 MM	1 MM	2 MM
HLA-A	11	67	40
B	4	40	74
DR	9	53	56

Rejection was monitored by endomyocardial biopsy. Grading of the biopsies was according to Billinghams criteria of none, mild, moderate and severe rejection [26]. For the diagnosis of moderate rejection, the coexistence of myocyte necrosis and mononuclear infiltrates was required. In that case, anti rejection treatment was instituted, which consisted of bolus steroids or, in case of ongoing rejection, of a two weeks course of a polyclonal rabbit anti thymocyte globulin preparation. There were no cases of severe rejection. In the early posttransplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to once every four months at one year. After an acute rejection episode the next biopsy was taken one week following rejection therapy. Three patients who died within 3 weeks after transplantation (from other causes than severe rejection) were

excluded from this study. We received a total of 1285 endomyocardial biopsies, 4-22 from each patient (median 10).

HLA typing
Spleen cells or peripheral blood mononuclear cells (obtained by Ficoll separation of heparinized blood) were typed for HLA class I antigens according to the standard NIH lymphocytotoxicity assay, and typed for HLA-DR by the two-colour fluorescence assay with a set of highly selected antisera [27].

Culture method
Lymphocyte cultures were established from EMB as described previously [21]. In brief, each biopsy was divided into 2 or more fragments and placed into 2 or more wells of a 96 well roundbottom tissue culture plate (Costar 3799, Cambridge, MA) with 200 µl culture medium in the presence of 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. When growth was observed the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing down or cell death was observed the cultures were restimulated by adding either 10^5 irradiated (40 Gy) donor spleen cells/well or 5 x 10^3 EBV transformed donor cells/well (irradiated with 80 Gy). Only 6% of the cultures needed restimulation in order to obtain sufficient amounts of cells for a cell-mediated cytotoxicity assay.

Allogeneic stimulator/target cells. PHA-blasts were obtained by culturing spleen cells for at least 5 days in the presence of 1% PHA-M (Difco, Detroit, MI) and culture medium: RPMI 1640 + 5% pooled human serum and 5% lymphocult-T (Biotest). EBV transformed B cell lines were set up and cultured as previously described [28].

Phenotypic analysis
The phenotypes of the graft infiltrating lymphocytes were analysed by two colour flow cytometry after staining with monoclonal antibodies directed against CD8 (anti-leu2) and CD4 (anti-leu3), both purchased from Becton Dickinson, Mountain View, CA. The antibodies were directly conjugated to fluoresceine or phycoerythrin. A more extensive phenotypic characterisation of the cultured cells is described elsewhere [21]. A T cell subset was

considered to be predominant when it comprised more than 60% of the cells in a culture.

Cell-mediated cytotoxicity assays

Biopsy-derived bulk cultures were tested for donor directed cytotoxicity in a standard 4-h ^{51}Cr release assay according to the European Standard Technique [29]. As target cells we used donor derived cell lines and a panel of unrelated target cells (phytohaemagglutinin T cell blasts or EBV transformed B cell lines) sharing one or more HLA antigens with the donor, and a third party control. The specificity for donor HLA class I (HLA A and B) or class II (HLA DR) antigens was determined by testing the cytotoxicity of the biopsy-derived T cell lines against a panel consisting of 5-10 (median 7) target cell lines. Each individual HLA-antigen was represented 1-4 times in the cell panels. If the cytotoxicity against an HLA-antigen was difficult to interpret like the example of HLA-B15 in figure 1 (left panel), this antigen was considered to be not tested. The HLA-antigens studied in the present report are listed in table 2.

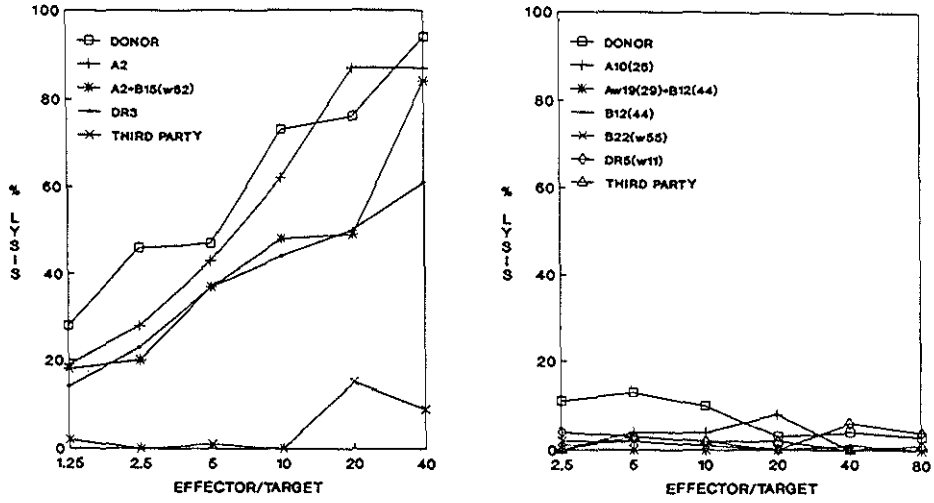

Figure 1 Representative example of a positive (left) and a negative cytotoxicity titration (right) of biopsy derived cultures from 2 different patients. The cultures were not restimulated with donor cells and were cultured for 40 and 26 days, respectively. In these examples, EBV transformed B cell lines were used as targets. The HLA-antigens shared by target cells and the heart donor are indicated. HLA-typing: left panel: Recipient: A1, A10(25), B18, B35, DR2(w15),DRw6(w13). Donor: A2, B15(w62), B18, DR2(w15), DR3. Right panel: Recipient: A2, A3, B35, B37, DR7, DRw10. Donor: A10(26), A19(29), B12(44), B22(55), DR5(11), DR7.

Table 2 CML reactivity of endomyocardial biopsy derived lymphocyte cultures against the most prevailing mismatched HLA-antigens, expressed as numbers and percentages of cultures reactive against an HLA-antigen.

	Numbers of cultures		
HLA-MM	tested	positive in CML (%)	No. of patients tested
HLA-A:			
A1	73	18 (25)	24
A2	90	56 (62)	23
A3	58	19 (33)	22
A10	38	7 (18)	12
A11	36	7 (19)	7
Aw19	119	32 (27)	29
HLA-B:			
B5	58	23 (40)	11
B7	46	21 (46)	19
B8	54	23 (43)	14
B12	68	38 (56)	19
B18	34	13 (38)	11
B27	21	11 (52)	7
B35	59	32 (54)	15
B40	37	9 (24)	15
B15	30	4 (13)	9
HLA-DR:			
DR1	85	35 (41)	20
DR2	42	17 (40)	16
DR3	62	31 (50)	19
DR4	72	33 (46)	17
DR5	87	32 (37)	18
DRw6	50	22 (44)	12
DR7	59	29 (49)	16
DRw8	37	10 (27)	10

2.5×10^3 ^{51}Cr labelled target cells were mixed with effector cells in 200 µl culture medium per well in 96 well U-bottom microtiter plates (Costar). Serial double dilutions with effector/target ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in 5% CO_2. Supernatants were harvested with a Skatron harvesting system (Skatron-AS, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA). According to the recommendations of the European CML Workshop [29], cultures were

considered cytolytic when the experimental lysis percentage exceeded 10% at an effector:target ratio of 20:1 or greater, and the slope of a graph was positive. Some representative cytotoxicity titrations are represented in figure 1. Series of double dilution studies revealed that lysis percentages of autologous control cell lines did not exceed 10%.

Statistical analysis

The significance of differences among the various groups of patients and cultures was analysed by Mann Whitney test or by X^2 test, respectively. Freedom from rejection rates were computed by actuarial methods and statistical significance was estimated by log-rank analysis.

Results

HLA-mismatches and acute rejection

In the DR matched patient group 56% of patients remained free from rejection at 6 months, compared to 29% of patients with 1 and 22% with 2 DR mismatches with their donors. For the combination of HLA B and DR antigens a significant effect on freedom of rejection was observed ($p < 0.05$, figure 2). Transplants with two or fewer mismatched HLA-B and DR antigens displayed a 14% higher freedom from rejection at 6 months compared to those with 3 or 4 HLA-B and DR incompatibilities. The number of HLA-A mismatches did not show any additive effect on freedom from rejection rates.

No significant relation between the number of acute rejection episodes and the number of mismatches on the individual A, B or DR locus was observed.

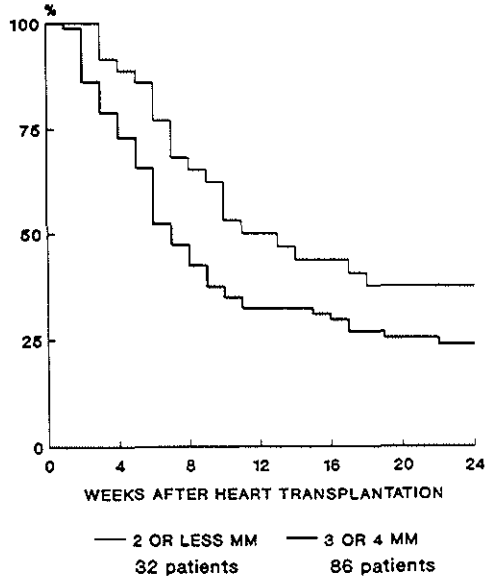

Figure 2

Actuarial freedom from rejection of heart transplants in relation to matching for HLA-B and DR antigens. The freedom from rejection of patients with ≤ 2 or > 3 mismatches for the combined B and DR antigens were 65% and 42% at two months ($p = 0.01$), and 37% and 24% at 6 months ($p = 0.05$), respectively.

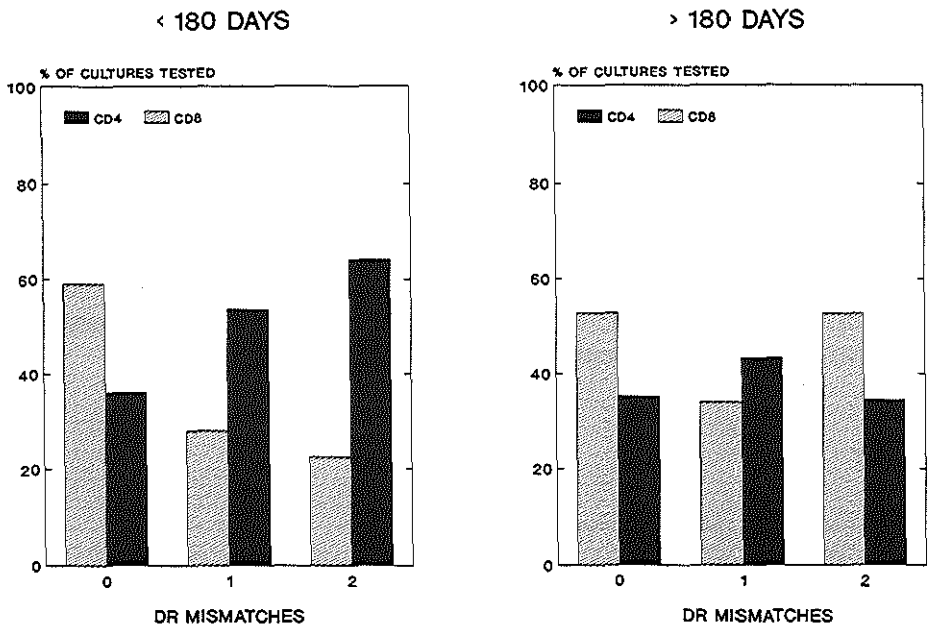

Figure 3 Predominant phenotype of endomyocardial biopsy derived lymphocyte cultures in relation to the number of HLA-DR mismatches between donor and recipient. In the first 180 days after transplantation, cultures from patients without DR mismatches with their donors were most often dominated by $CD8^+$ T cells ($p < 0.005$ compared with DR-matched combinations), while in cultures from patients with DR mismatches $CD4^+$ T cells comprised the predominant subset in the majority of cultures (left panel). After the first 6 post transplant months, no significant differences between the groups were found (right panel).
Numbers of cultures tested: Left panel: 0 DR MM (n = 22), 1 DR MM (n = 160), 2 DR MM (n = 226). Right panel: 0 DR MM (n = 17), 1 DR MM (n = 106), 2 DR MM (n = 110).

HLA mismatches and CD4/CD8 phenotypes

From all patients, approximately 60% of the endomyocardial biopsies yielded lymphocyte cultures. The success of culturing did not depend on the degree of HLA-DR matching between donor and recipient, since the mean percentages growing biopsies were highly comparable in patients with 0, 1 or 2 DR mismatches with their donors (57, 59 and 63%, respectively). In the first 180 days after transplantation, the number of HLA-DR mismatches between donor and recipient had a pronounced influence on the phenotypic composition of the EMB derived lymphocyte cultures (figure 3). Recipients with HLA-A and B mismatches but without DR-mismatches with their

donors yielded cultures that were dominated by $CD8^+$ T cells in 60% of cases ($p < 0.005$ compared to DR mismatched combinations). In cultures from patients with DR mismatches $CD4^+$ T cells comprised the predominant T cell subset. The predominance of $CD4^+$ T cells was most evident in patients with 2 DR mismatches with their donors ($p = 0.025$ compared with DR matched combinations). After the first 6 post transplant months, no significant differences between the groups were found.
The influence of the degree of HLA class I matching on growth and phenotypic composition of the cultures was hard to evaluate, because all patients except one had one or more class I mismatches. The EMB derived cultures ($n = 7$) from this single patient were all dominated by $CD4^+$ T cells. In the remaining patients, no significant relation was found between the number of HLA class I mismatches and the degree of CD8 predominance.

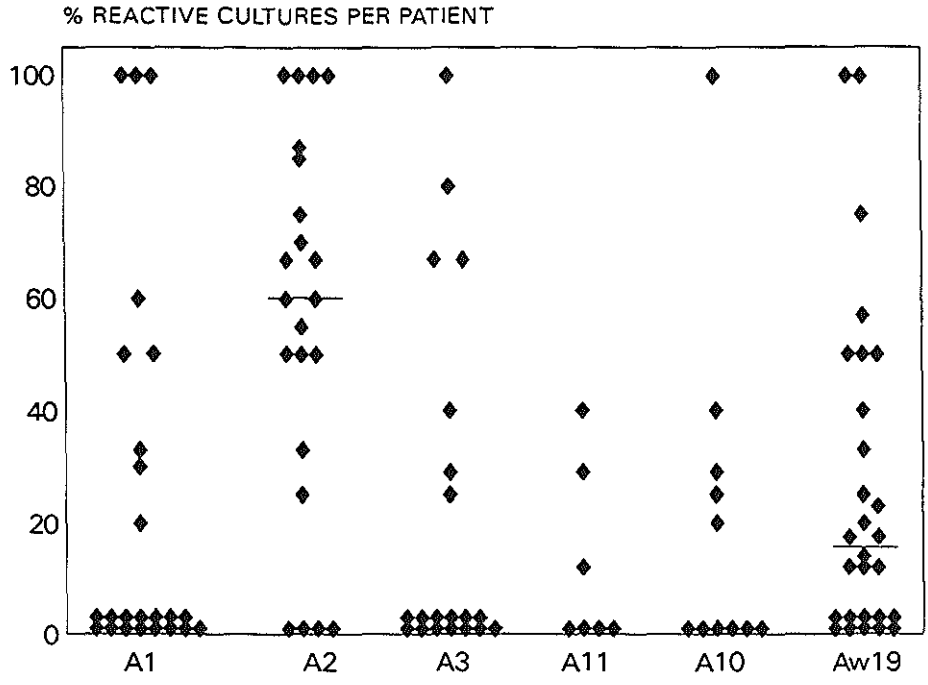

Figure 4 Percentage cytotoxic cultures against individual mismatched HLA-A antigens. Each dot represents one patient. From each patient, 1-12 (median 4) biopsy derived cultures could be tested. Against an HLA-A2 mismatch a significantly higher percentage reactive cultures was found when compared with other mismatched HLA-A antigens ($p < 0.001$, Mann-Whitney test). Medians of a group are indicated as horizontal lines.

Table 3 CML reactivity against mismatched HLA-B and DR antigens, expressed as median percentage reactive cultures per patient group with this HLA-incompatibility. From each patient, 1-12 (median 4) biopsy derived cultures could be tested.

	Median percentage reactive cultures
HLA-B:	
B5	22
B7	50
B8	38
B12	60
B18	60
B27	50
B35	60
B40	0
B15	0
HLA-DR:	
DR1	45
DR2	23
DR3	40
DR4	67
DR5	33
DRw6	22
DR7	40
DRw8	6

Table 4 CML specificity of EMB derived cultures against panel cells sharing mismatched HLA A, B or DR antigens with the donor. Relation with time after transplantation.

	numbers of reactive cultures		
CML specificity	< 180 days n (%)[1]	> 180 days n (%)	p. value[2]
HLA-A	79 (38)	28 (33)	n.s.
HLA-B	127 (57)	38 (40)	< 0.01
HLA-DR	120 (55)	34 (37)	< 0.01

In the first half year, the incidence of HLA-A directed cytotoxicity was significantly lower than that against B or DR antigens ($p < 0.001$).

[1] Number and percent of reactive cultures.

[2] X^2 test.

Cytotoxicity

In vitro studies showed that the majority (n = 234, 75%) of the tested endomyocardial biopsy derived T lymphocyte cultures (n = 324) was cytotoxic against donor antigens. Of these cultures, 165 (53%) and 154 (49%) were cytotoxic against HLA-B and DR antigens, respectively. Significantly fewer cultures (n = 107, 36%, $p < 0.005$) showed cytotoxicity against HLA-A antigens. This higher immunogenicity of HLA-B and DR antigens was also apparent when we analyzed the CTL reactivity against several individual HLA-antigens (table 2). This was analyzed for broad specificities, and not splits. Against most HLA-A antigens a low percentage of reactive cultures was found. Only HLA-A2 was found to be of comparable immunogenicity to HLA-B and DR antigens. Sixty two % of 90 tested cultures from 23 donor recipient combinations with an HLA-A2 incompatibility showed reactivity against A2, which was significantly higher than generally found against other HLA-A antigens ($p < 0.001$, table 2). When analyzed as the percentage reactive cultures for each individual patient, a median of 60% HLA-A2 reactive cultures was found, which was again significantly higher than against other mismatched HLA-A antigens ($p < 0.001$, figure 4). Among the HLA-B antigens, no evidence for such an immunodominant locus-allele was found. Generally, a high percentage of tested cultures showed cytotoxicity against mismatched HLA-B antigens, while against some of these antigens the reactivity was lower (table 2, 3). A similar pattern was found for reactivity against HLA-DR mismatches (table 3).

In time, donor directed cytotoxicity declined significantly ($p < 0.03$) from 79% (181 of 229) of the cultures established from EMB taken in the first 180 days, to 61% (58 of 95) after 6 months. This was due to a fall in the percentage of cultures that were cytotoxic against mismatched donor HLA-B and DR antigens (table 4).

A higher number of HLA-A, B or DR mismatches between donor and recipient was found to be positively correlated with the percentage of cytotoxic cultures directed against these antigens (table 5). Biopsies from patients with two A, B or DR mismatches with their donors yielded a higher proportion of cytotoxic cultures directed against these antigens when compared to EMB from patients who had only one mismatch for any of these HLA antigens. The pronounced dose effect of HLA-A mismatches was mainly found when the second HLA-A antigen was A2 (in 62% of cultures from donor-recipient combinations with 2 HLA-A mismatches and a positive CML against HLA-A, compared with only 28% of cultures not reactive against HLA-A; $p < 0.001$, X^2 test).

Table 5 The dose effect of the number of HLA mismatches per locus on CML reactivity of endomyocardial biopsy derived cultures.

	numbers of reactive cultures		
CML specificity	1 mismatch n (%)[1]	2 mismatches n (%)	p. value[2]
HLA-A	86 (34)	77 (52)	0.005
HLA-B	78 (50)	145 (60)	0.055
HLA-DR	73 (40)	119 (52)	0.025

[1] Number and percent of reactive cultures.
[2] X^2 test

Discussion

The present study shows that the number of mismatched HLA-B and DR antigens on a transplanted heart, but also HLA-A antigens, is positively correlated with the percentage of cytotoxic endomyocardial biopsy derived cultures directed against these mismatched HLA antigens. The incidence of HLA-A directed cytotoxicity was, however, lower than that directed against B- or DR-mismatches. This apparently high immunogenicity of HLA-B and DR antigens may account for the significantly lower freedom from rejection rates in the patient group with more than 2 HLA-B and DR mismatches. This association between the number of HLA-B and DR mismatches and freedom from rejection was also described by others [13]. Studies on the effect of matching for HLA antigens in renal [3-8] and heart transplantation [10,11] showed that matching for HLA-B and DR has a significant influence on graft survival. In normal individuals HLA-B antigens are more immunogenic to cytotoxic T cells than HLA-A antigens, although major individual differences were found in the frequency of alloreactive CTLp directed against HLA-class I [31-33] and class II [34] antigens. Until now a direct correlation between pretransplant CTLp frequencies and transplant outcome has not been shown yet. But it was found in mice that an increase of CTLp frequency after transplantation is associated with allograft rejection [35]. In renal transplant patients a decrease of donor specific CTLp is correlated with good graft function [36], and a low patient specific CTLp frequency in a bone marrow donor gives less graft versus host disease [37].
In the present study, a "dose effect" phenomenon of the number of mismatches on CML-reactivity was found for HLA-A, B and DR antigens. This dose effect phenomenon of HLA mismatches could also be observed in the higher 6 month freedom from rejection in patients with 2 or fewer HLA B and DR mismatches in the first half year. This is consistent with data of

Opelz [3, 10, 38], who showed that during the early post transplant course, HLA-B and DR mismatches exerted a strong influence on transplant survival, in contrast to mismatched HLA-A antigens. However, the influence of the latter on long-term survival was comparable to that of HLA-B and DR antigens [38]. Interestingly, the dose effect for HLA-A mismatches was mainly found when the second mismatched antigen on the A locus was A2.
Our finding of high reactivity against HLA-A2 antigens expressed on donor cells confirms the observation of others [33, Roelen, personal communication], that HLA-A2 is an immunodominant locus allele. They found high anti-A2 CTL precursor frequencies among normal individuals and in highly sensitized patients awaiting renal transplantation, respectively. By inhibition experiments with CD8 monoclonal antibodies it was shown that all anti-HLA-A (including A2) precursor CTL could be inhibited, indicating that these cytotoxic T cells have a low avidity for HLA-A antigens. Of HLA-B directed CTL, significantly fewer could be inhibited. In previous reports [39, 40] we have shown that low avidity alloreactive CTL are probably not relevant for the rejection process. High avidity alloreactive CTL can be demonstrated in the peripheral blood of highly sensitized candidates for renal (re-)transplantation, and in the grafts of rejecting heart transplant recipients. Interestingly, preliminary data from Eurotransplant (Thorogood) show that a mismatch for HLA-A2 does not result in a lower kidney transplant survival. In the present study this could not be evaluated, because all patients also had several HLA-B and DR mismatches with their donors.
In the 1 and 2 DR mismatched groups significantly more CD4-dominated cultures were derived from the biopsies than in the DR-matched group. $CD4^+$ cells are known to be of crucial importance in the initiation of rejection [14-20]. Interaction of these cells with donor class II MHC antigens expressed on the graft tissue and on passenger leucocytes of donor origin results in activation of $CD8^+$ cells which recognize MHC class I antigens. Both $CD4^+$ and $CD8^+$ cells play a role in the rejection of mismatched grafts [14-16]. Rejection of class I disparate grafts appears to be most dependent on $CD8^+$ cells, although $CD4^+$ cells can be activated as well via presentation of donor MHC class I antigens on recipient antigen presenting cells in the context of self-HLA class II molecules [41,42].
More than 180 days after transplantation a decline in the number of the CD4 dominated cultures was found. This may be due to a lower expression of donor type class II antigens on graft tissue, due to the replacement of donor dendritic cells by the patients' antigen presenting cells [43,44]. As a consequence fewer class II specific $CD4^+$ lymphocytes may be attracted to the graft. There are data from animal heart transplant models that show that this reduction of the number of HLA-class II expressing dendritic cells may already start early after transplantation, which may explain our finding that

in some patients an earlier decline of DR directed cytotoxicity is found [45] (data not shown).
Lower expression of donor type HLA-antigens on graft tissue may also play a role in the lower incidence of cytotoxicity directed against these antigens after 6 months [47,48]. Next to the influence of HLA expression on the graft, other mechanisms may also be involved. Specific suppression of anti-donor responses by regulatory T cells, clonal anergy or deletion of anti-donor T cells, or down modulaton by anti-idiotypic antibodies are thought to be important mechanisms contributing to 'acquired immune tolerance in human transplant recipients' [21, 36, 48-50].
In conclusion, we showed that the number and nature of HLA mismatches between donor and recipient strongly influence the cellular immune response within the transplanted heart, resulting in a significant effect on freedom from rejection.

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CHAPTER 6

ALTERED SPECIFICITY OF ALLOREACTIVE CARDIAC GRAFT INFILTRATING CELLS BY PROPHYLACTIC TREATMENT WITH OKT3 OR H-ALG[1]

Alice J. Ouwehand, Carla C. Baan, Kees Groeneveld, Aggie H.M.M. Balk, Nicolet H.P.M. Jutte, Egbert Bos, Frans H.J. Claas and Willem Weimar.

Abstract

Graft infiltrating lymphocytes from patients who were prophylactically treated with OKT3 or horse antilymphocyte globulin (H-ALG) were found to have different specificity patterns from those in the control group that received cyclosporine (CsA) from the day of transplantation. This prophylactic treatment led to a significant decrease of the HLA-DR-directed cytotoxicity, while the cytolytic response against HLA-class I mismatches was hardly affected. In H-ALG patients without rejection, the percentages of class I reactive cultures were found to be lower than in the other treatment groups, which was mainly due to a lower percentage of HLA-B-reactive cultures. In CsA and OKT3 patients cytotoxic T cells were rather directed to HLA-B mismatches than to HLA-A antigens, while in H-ALG patients no difference in HLA-A and B directed cytotoxicity was found. Our data suggest that OKT3 and H-ALG influence the specificity of the T cell allorepertoire, resulting in a decreased frequency of class II-specific cytotoxic T cells after transplantation. H-ALG also has a downregulating influence on

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the CTL response against HLA class I (HLA-B) antigens. In some patients a fast regeneration of these cells occurs, which results in a higher rejection incidence during the first posttransplant year.

Introduction

Prophylactic treatment with monoclonal and polyclonal heterologous immunoglobulins directed against lymphocytes or thymocytes has been suggested to have a beneficial effect on freedom from rejection and rejection incidence after transplantation (1-4). Such treatment dates back to the sixties, when Monaco and Medawar reported that antilymphocytic antisera can abrogate an alloimmune response. However, the immune response against an allograft always regenerated after some time (5-8) although the specificity of such a newly regenerated alloimmune response remained elusive.
Controlled studies with prophylactic polyclonal and monoclonal antilymphocyte antibody preparations provide a perfect opportunity to study the effect of these agents on the specificity of the regenerating alloreactive immune response, because comparison is possible with a control group receiving only cyclosporine (CsA). In our centre, two randomized controlled trials have been performed among heart transplant recipients. In the first trial, patients were randomly allocated to Orthoclone OKT3 or to CsA therapy from the day of transplantation (9). In another controlled study, OKT3 prophylaxis was compared with horse antilymphocyte globulin (H-ALG) (10).
In a previous report we showed that the cytotoxic capacity and specificity of graft infiltrating cells derived from endomyocardial biopsies (EMB) is related with rejection (chapter 3, this thesis). We also showed that donor HLA-B and DR antigens are important targets for cytotoxic graft infiltrating T lymphocytes, and that a higher number of HLA-B and DR mismatches has a negative influence on freedom from rejection (11). In the present study we investigated the developing alloreactive T cell repertoire in the graft after induction therapy with OKT3 or H-ALG, and this was compared with a control group receiving CsA from the day of transplantation.

Materials and methods

Patients
We studied biopsy-derived graft infiltrating cells from 92 consecutive heart transplant recipients transplanted between January 1988 and January 1991. These patients were followed from the day of transplantation. Three patients who died within 3 weeks after transplantation were excluded from this study.

All patients had received preoperative blood transfusions and all received cyclosporine (Sandimmun^{R}, Sandoz, Basel, Switzerland) and low-dose prednisone as maintenance immunosuppression. The patients studied were included in 2 randomized controlled studies: In the first trial patients were allocated to either CsA therapy from the day of transplantation or to a 7-day course of Orthoclone OKT3 (5 mg/kg/day; Ortho Pharmaceutical Corp, Raritan, NJ), while oral CsA was introduced on the fifth postoperative day. The control group received CsA starting just before operation. Steroids were administered in the same dosage in both groups (9). In the second trial patients were allocated to OKT3 as described for the first trial, or to a 3-7-day course of H-ALG (Institut Merieux, Lyon, France) in a dosage of 425 lymphocytotoxic units (0.5 ml) per kg of body weight per day.

The actuarial patient survival was comparable in all treatment groups (88-91% at 4 years), and no significant difference in actuarial freedom from rejection (27-28% at 6 months) or rejection incidence (0-6, median 1) was observed.

The mean number of HLA mismatches between donor and recipient was 1.30, 1.55, and 1.39 for the A, B, and DR-antigens, respectively. Only one patient was fully matched for HLA-A and B, and 7 patients for HLA DR antigens.

Detection of acute rejection was performed by endomyocardial biopsy. Grading of the biopsies was according to Billingham's criteria of none, mild, moderate, and severe rejection (12). For the diagnosis of moderate rejection, the coexistence of myocyte necrosis and mononuclear infitrates was required. Antirejection treatment was instituted in case of moderate rejection, and consisted of bolus steroids or, in case of ongoing rejection, with a 2-week course of a polyclonal rabbit antithymocyte globulin preparation. There were no cases of severe rejection.

In the early posttransplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to once every 4 months at 1 year. To evaluate the effect of the prophylactically given anti-T cell antibodies on alloreactive graft infiltrating lymphocytes derived from the EMB, we censored the data after the first rejection, and analyzed only the data when no antirejection therapy had been given.

HLA typing

Spleen cells or peripheral blood mononuclear cells (obtained by Ficoll separation of heparinized blood) were typed for HLA class I antigens according to the standard NIH lymphocytotoxicity assay, and typed for HLA-DR by the two-color fluorescence assay with a set of highly selected antisera (13).

Culture method

Lymphocyte cultures were established from EMB as described previously (chapter 2, this thesis). In brief, each biopsy was divided into 2 or more fragments and placed into 2 or more wells of a 96 well round bottom tissue culture plate (Costar 3799, Cambridge, MA) with 200 µl culture medium in the presence of 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. When growth was observed, the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing or cell death was observed, the cultures were restimulated by adding either 10^5 irradiated (40 Gy) donor spleen cells/well or, when available, 5 x 10^3 EBV (Epstein Barr Virus) transformed donor cells per well (irradiated with 80 Gy). Donor cells were rarely (only in 6% of cultures) added in the first 5 weeks of culture.

Allogeneic stimulator/target cells

Phytohaemagglutinin-blasts were obtained by culturing spleen cells for at least 5 days in the presence of 1% PHA-M (Difco, Detroit, MI) and culture medium: RPMI 1640 + 5% pooled human serum and 5% Lymphocult-T (Biotest). EBV-transformed B cell lines were derived as previously described (chapter 2, this thesis).

Cell-mediated cytotoxicity assays

A standard 4-hr ^{51}Cr release assay was used to measure the cytotoxic capacity of the cultures against donor cells and a panel of unrelated target cells sharing one or more HLA antigens with the donor (chapter 2, this thesis). ^{51}Cr labelled target cells (2.5 x 10^3) were mixed with effector cells in 200 µl culture medium per well in 96 well U-bottom microtiter plates (Costar). Serial double dilutions with effector/target ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in 5% CO_2. Supernatants were harvested with a Skatron harvesting system (Skatron-AS, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter. Cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector/target ratio of 20:1 or greater, and the slope of a graph was positive. Series of double dilution studies revealed that lysis percentages of autologous controls did not exceed 10%.

Statistical analysis

The significance of differences between the different treatment groups was analyzed with the Mann-Whitney test. Freedom from rejection rates were computed by the Kaplan Meier test, and statistical significance was estimated by log-rank analysis.

Results

Influence of the immunosuppressive treatment on the specificity of donor-reactive cultures

The specificity of the cytolytic biopsy-derived cultures proved to be influenced by the immunosuppression that the patients had received during the first postoperative week. We analyzed the data in the period before the first rejection treatment. For nonrejecting patients, day 61 after transplantation was taken as cut-off point (the mean follow-up in the rejectors before their first rejection). At this time, 80% of the rejectors had had their first rejection.

During this period, we received a total of 552 EMB from the 92 heart transplant recipients. From 331 (60%) EMB (from 89 patients) we were able to grow lymphocyte cultures, of which 231 (from 79 patients) yielded sufficient cells for testing cytolytic function.

The success of culturing did not depend on the immunosuppressive agents used, since the percentages growing biopsies per individual patient were higly comparable in patients receiving CsA, OKT3, or H-ALG (median individual growth percentages of 58%, 57%, and 63%, respectively). Figure 1 shows how the actual number of growing cultures was distributed among the patients in the different treatment groups.

Seventy-four of the 79 tested patients supplied at least one biopsy that contained donor-reactive cytotoxic T cells; from the remaining 5 patients the cultures were not cytotoxic. From each patient a median number of 2-3 (range 1-7) EMB could be tested, independent of the nature of the prophylactic treatment or rejector or nonrejector status of the patient. There were 43 patients from whom 3 or more cultures were tested for cytolytic function (9 of 19 patients who received CsA, 20 of 37 with OKT3, and 14 of 23 with H-ALG). From each of the remaining 36 patients, 1 (17 patients) or 2 cultures (19 patients) could be tested.

The median culture period required for the generation of sufficient cells for a cell-mediated cytotoxicity assay (at least 10^6 cells) was 35 days (range 16-90 days). Only 7 of 120 cultures that were tested within 36 days of culture were restimulated with irradiated donor cells. In total, 86 of the 231 (37%) cultures needed restimulation in order to generate high numbers of cells.

Within a culture period of 3 months, no effect of this procedure on CML specificity could be demonstrated after repeated testing.
In the period under study, before the first rejection treatment, we found significantly less cytotoxicity directed against DR mismatches in the OKT3 and H-ALG groups than in the patients who received CsA from the day of transplantation ($p < 0.02$, figure 2).
Cytotoxicity directed against HLA class I antigens was much less affected. The incidence of cytolytic activity against HLA-B antigens was generally higher than against HLA-A antigens (table 1), except for the H-ALG patients, in whom the percentages HLA-A and B-directed cytolytic cultures were found to be comparably low. The lower incidence of HLA-B-directed cytotoxicity in the H-ALG group was mainly found in patients without acute rejection episodes ($p < 0.01$ when H-ALG nonrejectors were compared with nonrejectors on CsA or OKT3).

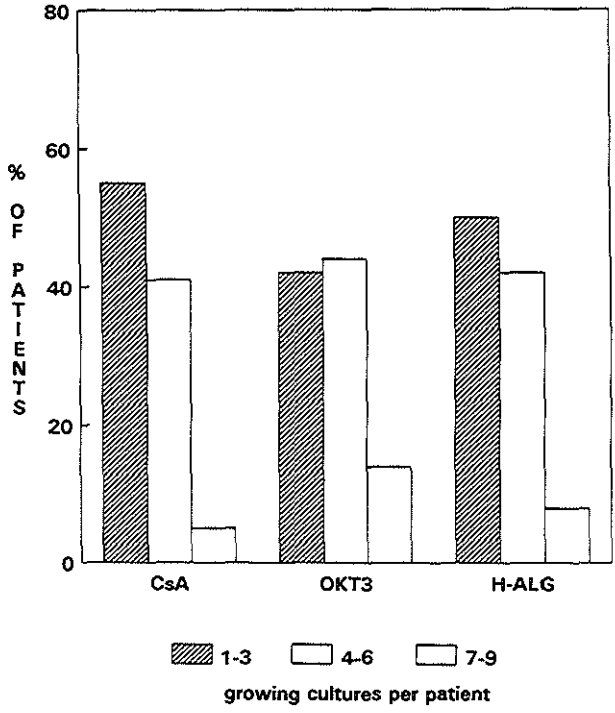

Figure 1 Distribution of the number of growing cultures in the period before the first anti rejection treatment among patients who received CsA (n = 19), OKT3 (n = 37) or H-ALG (n = 23) as prophylactic treatment. No significant differences were found between the different treatment groups in the number of successful cultures established from endomyocardial biopsies (Mann-Whitney test).

Table 1 Donor-directed cytotoxicity in the period before the first antirejection therapy.

	Median % reactive cultures per patient			
	A	B	DR	No. patients
CsA	59	100	75	19
OKT3	38	71	42	37
H-ALG	60	50	50	23

Median individual percentages of cytotoxic cultures directed against HLA-A, B, and DR mismatches in patients receiving prophylactic OKT3 or H-ALG, compared with patients who had received CsA from the day of transplantation.
Mann-Whitney test differences between the treatment groups: HLA-A, ns. (not significant); HLA-B, ns.; HLA-DR: CsA vs OKT3, $p = 0.01$, CsA vs H-ALG, $p = 0.03$.
Significant differences within the treatment groups: CSA: A vs B, $p = 0.05$; OKT3: A vs B, $p = 0.02$, B vs DR, $p = 0.08$; H-ALG: no significant differences.

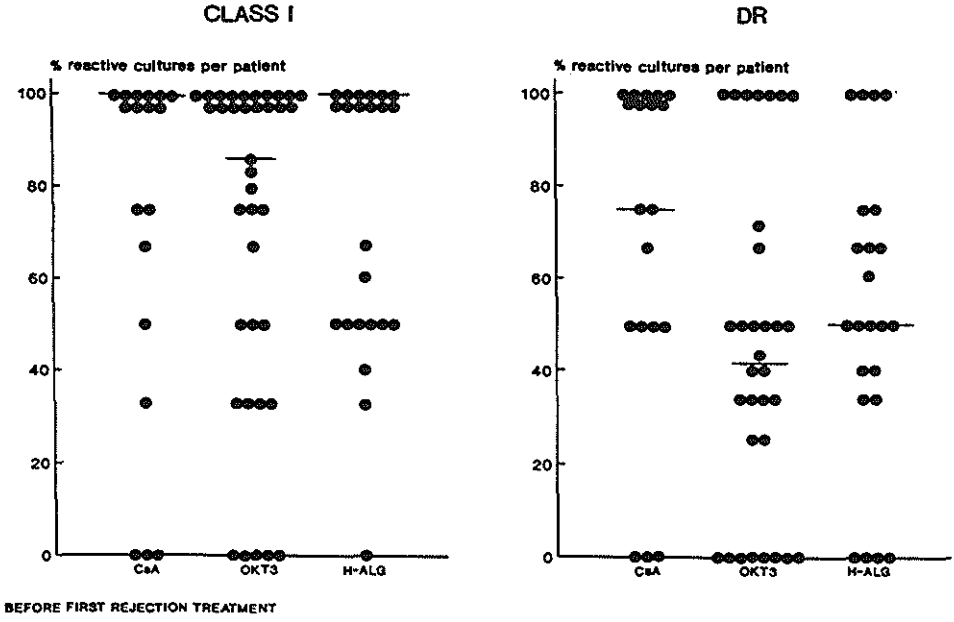

Figure 2 Percentage of HLA-class I (left) and HLA-DR-reactive cultures (right) in the period before the first rejection treatment in patients who had received CsA (n = 19), OKT3 (n = 37), or H-ALG (n = 23) as prophylactic therapy. Each dot represents the percentage reactive cultures of one patient. Median values are indicated as horizontal lines.
Mann-Whitney test: (left) No significant differences. (Right) CsA vs OKT3, $p < 0.02$; CsA vs H-ALG, $p = 0.07$.

Relation between donor-directed cytotoxicity and rejection

To ascertain the clinical relevance of our results, we investigated whether the percentage of tested cultures reactive against HLA class I or class II mismatches with the donor was correlated with the number of acute rejection episodes in the first year. Figure 3 shows that the number of acute rejections in the first year is correlated with a higher percentage of HLA class I-directed cytolytic cultures in the period before the first rejection treatment ($p = 0.08$). This is not the case for cytolytic activity against DR mismatches. The greatest contribution to the increased class I directed cytotoxicity in patients with rejection was made by reactivity against HLA-B antigens (medians of 50%, 75%, and 100% reactive cultures in patients with 0, 1 or 2 or more rejections, respectively).

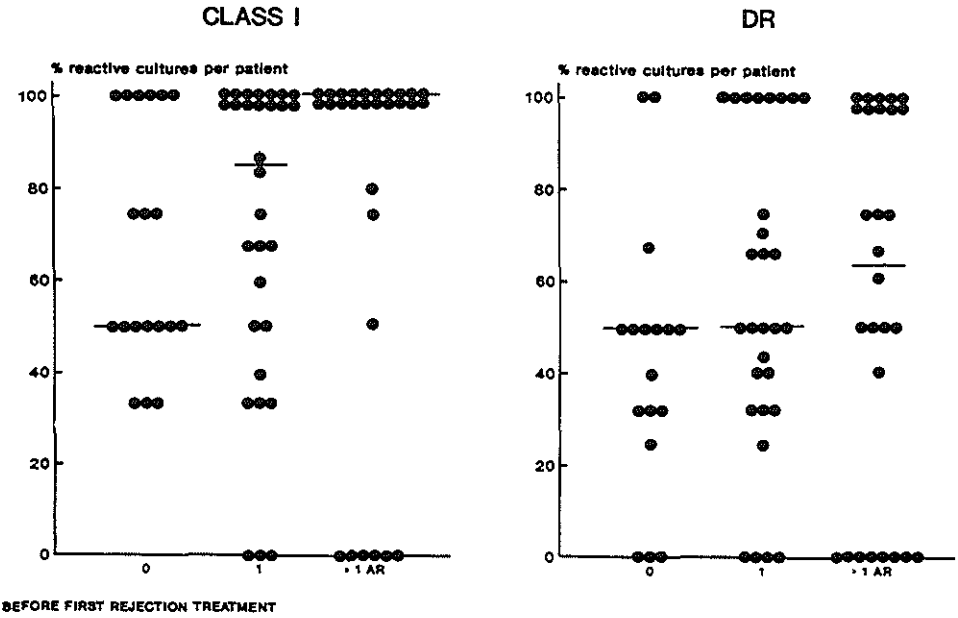

Figure 3 Percentage of cultures reactive against HLA-class I (left) and HLA-DR (right) mismatches in the period before the first rejection treatment in patients with 0 (n = 19), 1 (n = 30), or >1 (n = 30) acute rejections (AR) in the first year. Each dot represents the percentage reactive cultures of one patient. Median values are indicated as horizontal lines.
Mann-Whitney test: (left) 0 vs > 1 AR (acute rejections), $p = 0.08$; (Right) No significant differences.

The relation between CML specificity and rejection incidence in the different treatment groups

The relation between cytotoxicity directed against HLA class I mismatches in the early post transplant period (before the first rejection treatment) and

rejection incidence as described above was only statistically significant in the patients who had received prophylactic H-ALG: the rejectors yielded significantly higher percentages of HLA class I-reactive cultures (median 100% per patient) when compared with patients without rejection in the first year (a median of 50% cytotoxic cultures, $p = 0.01$, figure 4). This was caused by an increase in both HLA-A and HLA-B-directed cytotoxicity (HLA-A: median 25% in the nonrejectors and 67% in rejectors; HLA-B: from 0% to 100%). In the nonrejectors who had received H-ALG, the HLA-B-directed cytotoxicity was found to be significantly lower than in the other treatment groups ($p < 0.01$), while in the rejectors similar median percentages of HLA-B-directed cultures were found in all treatment groups. In H-ALG patients, also the median percentage of cultures with cytolytic activity against DR antigens was found to be related to the number of rejections later in the first year (medians of 64% in patients with rejection vs. 33% in patients without rejection, $p = 0.04$).

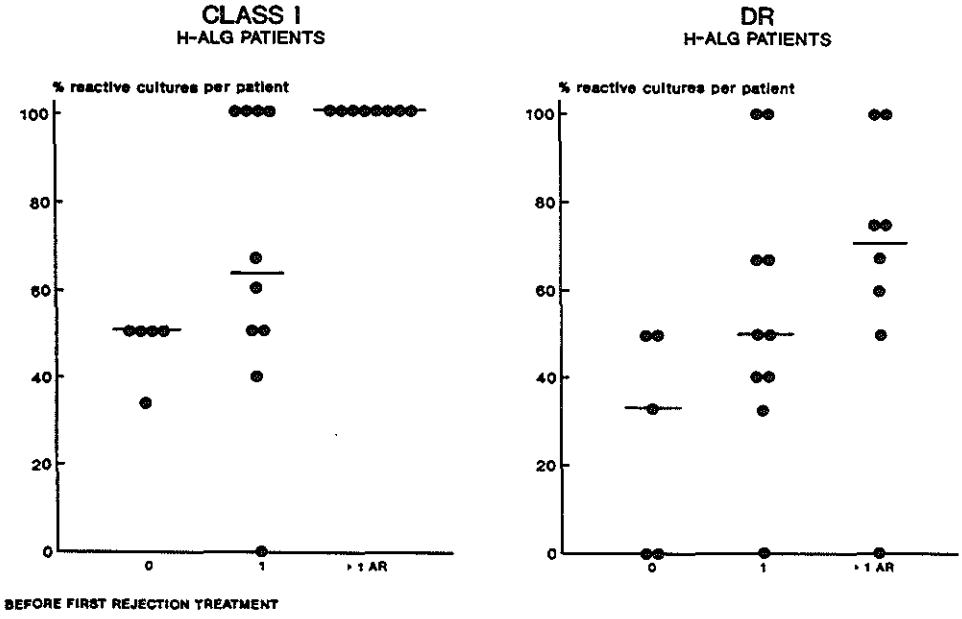

Figure 4 Donor class I (left) and DR directed cytotoxicity (right) in the period before the first rejection treatment in 23 patients who received prophylactic H-ALG. Relation to the number of acute rejections (AR) in the first year. Each dot represents the percentage reactive cultures of one patient. Median values are indicated as horizontal lines.
Mann-Whitney test: (left) 0 vs $\geq$ 1 AR, $p = 0.01$; 0,1 vs >1 AR, $p < 0.002$. (Right) 0 vs $\geq$ 1 AR, $p = 0.04$; 0 vs $\geq$ 2 AR, $p = 0.03$.

Our data show that prophylactic therapy with OKT3 or H-ALG has a downregulating influence on the cytolytic response of graft infiltrating cells against DR antigens expressed on the transplanted heart, while HLA class I-directed cytotoxicity is less affected.
Antilymphocyte immunoglobulins such as OKT3 and H-ALG are thought to eliminate the patients' T cell alloimmune repertoire that has been built up during their life times. Already in the sixties, Monaco and Medawar showed that antilymphocytic antisera can (temporarily) eliminate the immunological response to recall antigens (5-8). After such treatment the patient has to acquire a new T cell repertoire in the presence of a transplanted organ. This may result in another composition of the HLA-A, B, and DR-directed T cell response in comparison with patients who received CsA from the day of transplantation.
The presence of HLA class I-directed cytotoxic cells in cultures from biopsies taken prior to the first rejection treatment was associated with a higher number of rejections in the first year, while CTL reactivity against HLA-DR was not. However, the fact that the patients without DR mismatches had a higher freedom from rejection (55%) than patients with 1 or 2 DR mismatches (25%) (chapter 3, this thesis) shows that the DR alloantigens do play a role in the initiation of rejection. The mechanism by which this happens is thought to be mediated by recipient $CD4^+$ regulatory T lymphocytes that interact with class II antigens expressed on graft tissue and on passenger leucocytes of donor origin. This results in activation of $CD8^+$ cells that recognize HLA class I antigens (14-16). So it might well be that $CD4^+$ T helper activity does show a positive correlation with rejection, and that the higher level of HLA class I-directed cytolytic reactivity in patients who will develop one or more rejections is the consequence of this activity.
Both OKT3 and H-ALG have a downregulating influence on the development of class II-specific (most likely $CD4^+$) CTLs after transplantation, and herewith on the cytolytic reactivity against HLA-DR antigens expressed on the allograft. In addition to this phenomenon, H-ALG also inhibited the $CD8^+$ CTL response in some patients, which resulted in a lower reactivity against HLA-class I (HLA-B) mismatches. In the H-ALG-treated patients a higher percentage of donor HLA class I and also class II directed cytotoxic cultures was associated with a higher rejection incidence. In the OKT3 and CsA patients this was not found. One may speculate that after ALG therapy, which is more broadly directed than OKT3, both the ($CD4^+$) helper/$CD8^+$ CTL response and the ($CD4^+$) helper/$CD4^+$ CTL response are inhibited, but that in high responding patients a fast regeneration of these cells occurs, which results in a higher rejection incidence.

Only in the CsA group did we find that in the first posttransplant half year, both HLA-B and DR antigens are important targets for cytotoxic T lymphocytes. Studies on the effect of matching for HLA antigens in CsA treated renal transplant recipients showed that matching for HLA-B and DR has a benefical effect on transplant survival (17-21). In heart transplantation, we and others found that the combination of B and DR matching has a significant influence on graft survival (22, 23) and freedom from rejection (11,24). Not confined to transplant recipients, several investigators found major differences among normal individuals in the frequency of alloreactive CTL precursors directed against HLA class I (25-27) and class II antigens (28). Generally CTL precursor frequencies against HLA-B antigens are higher than those against HLA-A antigens (25,27). The range of the class II-directed CTL precursor frequencies in relation to those against HLA-class I antigens in the normal population is not known. The fact that the relation between HLA-A and B-directed cytotoxicity in CsA and OKT3 patients remained comparable to that in healthy individuals suggests that these agents affect the immune response against HLA-A, B alloantigens in a proportionate manner, while this does not seem to be the case for H-ALG. In these patients the difference in immunogenicity between HLA-A and B antigens was not found.

In conclusion, in patients treated with OKT3 or H-ALG as prophylactic therapy in the early posttransplant period, cytolytic reactivity patterns of the allospecific graft infiltrating lymphocytes were different from that of CsA-treated patients. This shows that antilymphocyte immunoglobulins change the specificity of the alloimmune response of the patient, whereby in some patients, H-ALG appears to have a broader inhibitory effect than OKT3. Whether this has a positive influence on long term graft survival has to be investigated.

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CHAPTER 7

CYTOMEGALOVIRUS INFECTION AND ALLOSPECIFIC CYTOTOXIC ACTIVITY OF GRAFT INFILTRATING CELLS AFTER HEART TRANSPLANTATION[1]

A.J. Ouwehand, A.H.M.M. Balk, C.C. Baan, L.M.B. Vaessen, N.H.P.M Jutte, E. Bos, F.H.J. Claas, W. Weimar

Abstract

We have investigated whether CMV infection has an effect on donor directed cytotoxicity of graft infiltrating cells in human heart transplants. Our study group consisted of 89 heart transplant recipients. Thirtyeight (43%) showed signs of CMV infection, in 28 of them cytolytic activity of biopsy derived cultures could be tested during the infection. Eight patients had a primary, and 20 a secondary infection. We found that during CMV infection, both primary and secondary, a significantly higher proportion of the biopsy derived cultures showed cytotoxicity against donor HLA antigens (X^2 test: $p < 0.01$ in comparison with 51 patients without infection). This was most evident in patients with both infection and acute rejection episodes when compared to patients with only one of these complications. This suggests that one process amplifies the other with regard to the upregulation of alloreactivity within the transplanted heart. In secondary infections, only an increase of donor class I directed cytotoxicity was found, while in primary

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infections cytotoxicity against both donor class I and II antigens were increased ($p < 0.005$ when compared to secondary infection).

Introduction

Cytomegalovirus (CMV) is a common pathogen in transplant recipients, in whom it is able to cause life-threatening disease. Apart from inducing clinical symptoms and signs, CMV has been associated with allograft rejection and graft-versus-host disease (1-7). Although there is no doubt about the existence of such a mutual relationship, there is no consensus about the exact sequence in which CMV infection and these alloreactive reactions occur. In studies in mice it has been shown that infection with this virus can induce polyclonal activation of alloreactive cytotoxic T lymphocytes (CTLs) (8,9), and may thus enhance alloreactivity against an allograft (10). Furthermore, CMV is able to increase MHC class I expression on the surface of human cells (11,12), and the combination of this increased class I expression and the enhanced alloreactivity may result in allograft rejection. On the other hand, some investigators have proposed that the rejection process may facilitate CMV infection by upregulating MHC expression, as the virus, which exists in vivo as a $ß_2$ microglobulin (the light chain of the MHC class I molecule) coated particle, might use MHC class I molecules as a receptor to penetrate the cell (11,13). However, more recent evidence suggests that MHC molecules are not the CMV receptor. Nowlin and coworkers (14) have shown evidence of the presence of 34- and 32 kDa HCMV specific attachment receptors on several human cell types, which are induced by the virus. One may speculate that the expression of these receptors is greatly enhanced in the presence of both CMV infection and allograft rejection. It is also possible that rejection therapy enhances CMV infection by abrogating the cellular immune response against the virus (15,16).
The functional studies mentioned above concern anti-virus and virus-associated responses of human peripheral blood CTLs or mouse-spleen cells. In the present study we analyzed the effect of CMV infection on alloreactive CTL responses within the transplanted heart, and explored the mutual relationship between CMV infection and acute allograft rejection.

Materials and methods

Patients
We studied cytolytic reactivity of endomyocardial biopsy derived lymphocytes from 89 heart transplant recipients transplanted between

February 1988 and January 1991. In table 1 the characteristics of these patients are shown. The immunosuppressive regimen consisted of cyclosporin A (CsA, Sandimmune^{R}, Sandoz, Basel, Switzerland) and low dose prednisone. The dose of CsA was adjusted according to specific plasma-through levels. All patients received blood transfusions prior to transplantation.
Detection of acute rejection was performed by endomyocardial biopsy (EMB). EMB were taken at regular intervals after transplantation. Grading of the biopsies was according to Billinghams criteria (17). Acute rejection was diagnosed when both myocyte necrosis and mononuclear infiltrates were present.

Table 1 Characteristics of the patient groups with and without active CMV infection.

		Number of patients	
		with infection	without infection
sex	male	31	43
	female	7	8
median age	years	48	49
	range	14-62	15-60
≥ 1 AR*		28	39
Freedom AR at 26 weeks†		24%	23%
Mean No. mismatches			
	HLA-A	1.21	1.37
	B	1.53	1.55
	DR	1.47	1.29
CMV serostatus donor/recipient			
	neg/neg	4	19
	pos/neg	10	4
	?/neg	2	0
	neg/pos	5	8
	pos/pos	6	6
	?/pos	11	14

* AR = acute rejections.
† Actual freedom from rejection: the percentage of patients who remained free from acute rejections at 26 weeks after transplantation.

Virologic methods
The CMV serostatus of the transplant recipients was screened for anti-CMV IgG by an ELISA (18). Recipients with a pretransplant ELISA titer < 100 were considered to be CMV seronegative. Serum of organ donors was retrospectively screened for CMV IgG antibodies. All CMV-IgG seronegative

recipients received anti-CMV immunoglobulins (Cytotect^{R}, Biotest Pharma GmbH, Dreieich, FRG), irrespective of the CMV serostatus of the organ donor (19). Samples of urine, throat wash, and blood were collected for detection of CMV early antigen every 14 days for 3 months (20). When indicated, more specimens were obtained for diagnosis thereafter. Routine monitoring included serology using an indirect immunofluorescence assay for IgM antibodies (21) and an ELISA for IgG antibodies.

Definitions
Active CMV infection was defined as any appearance of CMV IgM antibodies, any isolation of CMV from urine, throat or blood and any demonstration of the antigen. A patient was considered to have latent CMV infection when the ELISA titer of CMV-IgG antibodies was greater than 100. An active CMV infection was considered to be completed when the anti-CMV IgM antibody titers had declined to undetectable levels or when no CMV-antigen could be demonstrated nor CMV could be isolated from urine, throat or blood. As a sign of the remaining latent CMV infection, the anti-CMV IgG antibody titer is > 100. CMV disease was defined as illness with 2 of the following signs: otherwise unexplained fever (> 38°C) for at least 2 consecutive days, gastrointestinal, lung or central nervous system involvement, leucocytopenia, thrombocytopenia, elevated serum alanine or aspartate aminotransferases (> 2 times normal). This viral syndrome had to be confirmed by concomitant detection of CMV antigens.

Lymphocyte cultures were established from EMB as described previously (22). In brief, each biopsy was cultured in a 96 well roundbottom tissue culture plate (Costar 3799, Cambridge, MA) with 200 µl culture medium per well, in the presence of 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.
When growth was observed the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing or cell death was observed the cultures were restimulated by adding either 10^5 irradiated (40 Gray) donor spleen cells/well or, when available, 5 x 10^3 EBV (Epstein Barr Virus) transformed donor cells/well (irradiated with 80 Gray). Donor cells were rarely (only in 6% of cultures) added before testing the cultures for donor directed cytotoxicity in a cell-mediated cytotoxicity assay.

Cell-mediated cytotoxicity assays

Biopsy-derived bulk cultures were screened for donor directed cytotoxicity in a standard 4-h ^{51}Cr release assay according to the European Standard Technique (23). As target cells we used donor derived cell lines and a panel of unrelated target cells (phytohaemagglutinin T cell blasts or EBV transformed B cell lines) sharing one or more HLA antigens with the donor, and a third party control. The specificity for donor HLA class I (HLA A and B) or class II (HLA DR) antigens was determined by testing the cytotoxicity of the biopsy-derived T cell lines against a panel consisting of 5-10 (median 7) target cell lines.

^{51}Cr labelled target cells (2.5×10^3) were mixed with effector cells in 200 µl culture medium per well in 96 well U-bottom microtiter plates (Costar). Serial double dilutions with effector/target ratios varying from 1.25:1 up to 80:1 were used. The plates were incubated for 4 hours at 37°C in 5% CO_2. Supernatants were harvested with a Skatron harvesting system (Skatron-AS, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA). According to the recommendations of the European CML Workshop (23), cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector:target ratio of 20:1 or greater, and the slope of a graph was positive. Series of double dilution studies revealed that lysis percentages of autologous control cell lines did not exceed 10%.

Target cells used for the cell-mediated cytotoxicity assays:

B lymphoblastoid cell lines originated from infection of fresh peripheral blood mononuclear cells or spleen cells with Epstein Barr Virus obtained from the marmoset cell line B95-8, as described by Moreau et al. (24). These B cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, England). B lymphoblastoid cell lines express a high level of both HLA class I and class II antigens.

T lymphoblastoid cell lines were obtained by culturing peripheral blood mononuclear cells or spleen cells in culture medium supplemented with 1% phytohaemagglutinin (PHA; Difco, Detroit, MI), a T cell mitogen. These cells express HLA class I antigens at high levels. HLA class II antigen expression is much lower. PHA blasts were used as targets for HLA class I directed cytotoxicity.

Statistical analysis

The significance of differences between the groups of patients was analysed with the Mann-Whitney test, or by X^2 test for differences between groups of cultures.

Results

Patient populations

The patients could be followed for 70 to 1104 days (median 604 days) after transplantation. Active CMV infection was diagnosed in 38 (43%) of them. In these 38 infected patients the first day of onset of infection was day 41 (median, range 7 - 314), the median day of the last virus antigen detection was day 228 (range 8 - 840).

After stratification for donor/recipient serostatus it was found that the negative/negative combination experienced significantly less infection than seronegative recipients of a heart from a seropositive donor ($p < 0.005$, X^2 test), or when compared to heart transplant recipients who were CMV seropositive ($p < 0.05$, X^2 test) (table 1). Primary infections were observed in 16 patients and secondary infections in 22. Two of the 36 recipients of an organ from a CMV IgG-seronegative donor had clinical symptoms and signs. Before transplantation, these 2 patients had been CMV-IgG seronegative. Of the remaining patients, 7 of 16 (44%) seronegative and 7 of 37 (19%) seropositive recipients had CMV-disease.

A total of 51 patients remained free from signs of active CMV infection and constituted the control group for the analysis of the cytotoxicity tests. No statistically significant difference in sex, age, number of HLA mismatches, incidence of rejection or freedom from rejection at 26 weeks was detected between the patients with and without active infection. The 19 seronegative recipients of hearts from seronegative donors who remained free from active CMV infection were also analyzed separately, as in these patients CMV transmission is practically unconceivable.

Alloreactivity of graft infiltrating cells and the influence of active CMV infection

For the present study, we analyzed the results of all EMB taken in the first year after transplantation. A total of 1300 EMB were cultured, which resulted in 490 cases (38%) in sufficient cells for a cell mediated lympholysis assay. There were 230 adequate cultures (39%) in patients with active CMV infection and 260 adequate cultures (37%) in patients without this infection.

When we compared the cytolytic activity of EMB derived cultures from the 19 seronegative/seronegative donor-recipient combinations who remained free from active infection with that from the remaining 32 patients without active CMV infection (other CMV donor/recipient combinations), we found no significant differences: Sixty nine percent of the 84 tested cultures from the negative/negative patient group showed donor directed cytotoxicity, compared with 77% in the other patients without active infection. Of these cultures, 60% and 71% were directed to HLA class I antigens in the

respective groups, and 46% and 50% to class II antigens. When we calculated the percentage reactive cultures for each individual patient, we also found a similar distribution of the percentages donor directed cultures in both patient groups. These highly comparable results allowed us to use all patients without active infection as controls, irrespective of their donor/recipient CMV-serostatus.

Of 28 patients with active infection we tested 163 EMB derived cultures (1-14 per patient, median 5), of which 84 were obtained during CMV infection (1-7 per patient, median 2). From the individual patients without acive infection, 1-10 biopsies (median 4) yielded sufficient cells for cell mediated lympholysis. During active CMV infection, a significantly higher percentage of donor reactive cultures was found compared to the group without infection (74/84, 88% vs 173/234, 74%, $p = 0.01$, table 2). This proved to be due to an increased frequency of HLA class I directed cytotoxicity (71/84, 85% vs 157/234, 67%, $p < 0.005$). No difference between the two groups was found for cytolytic activity against HLA class II antigens (37/78, 47% and 106/219, 48%, respectively). Before and after signs of active infection could be demonstrated, the percentage of donor (class I) reactive cultures was comparable to that in the control group in a comparable period after transplantation (table 2). To ascertain that the increased cytolytic reactivity during active CMV infection was not caused by a few patients who had provided the majority of the biopsies, we also calculated the percentage reactive cultures for each individual patient. Figure 1 shows that the HLA class I directed cytolytic cultures originating from biopsies obtained during infection were derived from 25 of the 28 tested patients, and that in each of these 25 patients, the majority of the T cell lines was reactive against class I antigens. This was not significantly influenced by the presence or absence of clinical signs of CMV infection.

In this group of 28 patients, 8 had a primary and 20 a secondary infection, of whom respectively 6 (75%) and 7 (35%) had clinical symptoms and signs. During both primary and secondary infection, a significantly higher proportion of the EMB derived lymphocyte cultures showed cytotoxicity against donor class I antigens compared to the control group (X^2 test: $p < 0.05$ and $p = 0.02$, respectively), while in primarily infected patients the percentage of class II reactive cultures was also significantly increased ($p < 0.005$ when compared to cultures from secondary infected patients, and $p < 0.05$ when compared to the control group) (table 3).

Table 2 Donor directed cytotoxicity of endomyocardial biopsy-derived cultures before, during and after active infection with CMV. This is compared to the control group without infection in a comparable period after transplantation.

	CML * specificity		
	donor n† (%)	class I n (%)	class II n (%)
before infection	62 (79)	57 (73)	43 (55)
control group (0-41 days postTX)	101 (71)	92 (65)	54 (41)
during infection	74‡ (88)	71§ (85)	37 (47)
control group (< 228 days postTX)	173 (74)	157 (67)	106 (48)
after infection	11 (65)	11 (65)	6 (35)
control group (229-365 days postTX)	17 (65)	15 (58)	9 (38)

* CML = cell mediated lympholysis
† n = number and percentage of reactive cultures
‡ $p = 0.01$ when compared to the control group (X^2 test)
§ $p < 0.005$ when compared to the control group (X^2 test)

In the control group EMB cultures were derived from a comparable period after transplantation, which was determined by assessing the median day of the first virus isolation or detection of CMV antigen or CMV-IgM (41 days after transplantation (postTX)) and the last day of virus-, CMV-antigen or antibody detection (median 228 days). Only biopsies taken in the first year after transplantation were studied. The periods under study in the control group are indicated in parentheses.

Table 3 Donor directed cytotoxicity of endomyocardial biopsy-derived cultures in primary and secondary CMV infection.

	CML* specificity		
	donor n† (%)	class I n (%)	class II n (%)
primary infection	27 (90)	26 (87)	21 (70)
secondary infection	47 (87)	45 (83)	16 (33)
control group	173 (74)	157 (67)	106 (48)

* CML = cell mediated lympholysis
† n = number and percentage of reactive cultures

In the control group, EMB taken within the first 228 days after transplantation were studied (median duration of active CMV infection, as defined by the presence of detectable levels of CMV antigen or CMV-IgM, or any isolation of the virus).

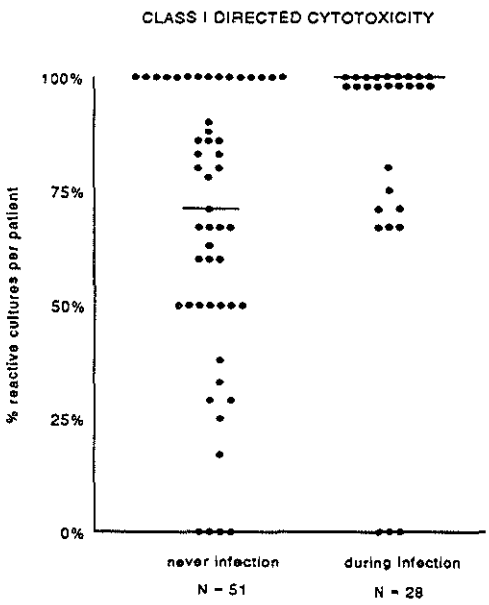

Figure 1

Percentage of cultures with cytotoxic reactivity against donor HLA class I antigens for individual patients with active CMV infection. During active CMV infection the infected patients yielded significantly higher percentages of reactive cultures compared to the control group who had never had this infection ($p = 0.008$, Mann-Whitney test). Median values are indicated as horizontal lines. Each dot represents one patient.

Active CMV infection and acute clinical graft rejection

The number of tested biopsies per individual patient was highly comparable in all groups, independent of whether they had experienced graft rejection or active CMV infection (table 4). In the patient groups with and without active infection, the percentage rejectors was comparable (table 1). Twenty nine of the 38 patients with active CMV infection (one without class I mismatches) experienced one or more acute graft rejection episodes during the period under study, in 22 of these 29 patients (76%) coincident with or immediately before the virus was detected. Infected patients with graft rejection more often had clinical signs of CMV infection than those without rejection episodes after transplantation (15 of 29 (52%) vs. 1 of 9 patients (11%), respectively; $p = 0.07$, X^2 test).

Table 4 Number of tested biopsy derived cultures per individual patient with and without rejection or CMV infection.

		No. of tested cultures per patient	
	No. patients	median	mean
with infection	38	4	5
AR^+	29	5	5,3
AR^-	9	4	3,9
never infection	51	5	4,5
AR^+	39	5	4,7
AR^-	12	4	4,3

In patients who experienced both rejection and infection the highest percentage of HLA class I directed cytolytic cultures was found when compared to patients who experienced rejection only ($p < 0.006$), or CMV infection only ($p < 0.03$) or to those who encountered neither of both complications (figure 2).
The median percentages of HLA class II directed cytotoxic cultures in individual patients were comparable in patients with or without CMV infection and/or rejection, and varied from 61% to 36% in the different groups (no statistically significant differences).

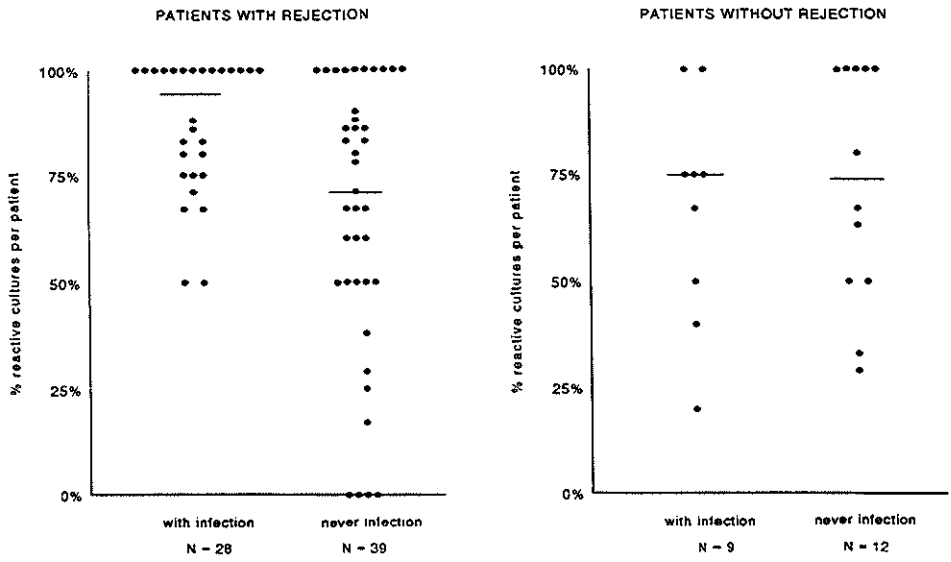

Figure 2 Influence of CMV infection on the individual percentage of cultures with donor HLA class I directed cytolytic reactivity in 67 patients with (left) and 21 patients without acute rejections (right) in the period under study. The HLA class I directed cytolytic activity was increased only in the group of patients with both acute rejection and infection ($p < 0.006$ when compared to rejecting patients without infection and $p < 0.03$ when compared to patients with infection and no rejection, Mann-Whitney test). Each dot represents one patient. Median values are indicated as horizontal lines.

Discussion

The early immune response against virus infections has been shown to be mainly cell mediated, and consists of antibody dependent cytotoxicity,

natural killer cell activity and MHC class I restricted $CD8^+$ cytotoxic activity (25,26). Of these, the virus specific T cell response has been characterized as the main protective cell-mediated immune response (27-30). In renal transplant recipients, a virus specific cytotoxic T lymphocyte (CTL) response was found to be an early manifestation of CMV infection, preceding virus excretion in several body fluids and a rise of the CMV-specific antibody titer (15).

Yang and coworkers (8,9) demonstrated in mice (not confined to transplantation), that virus infection induces polyclonal CTL activation, not only of CTLs directed against the challenge virus, but also of alloreactive CTL and memory CTL from a previous exposure to other viruses. This was thought to be mediated by lymphokines produced as a consequence of the virus infection. During infection, alloreactive CTL were found in high frequencies, and they appeared concomitantly with the virus specific CTLs. Concerning the human, many clinical studies are available on the relation between CMV infection and graft rejection. In contrast, there are only few data on how cellular alloreactivity within a transplanted organ is influenced by virus infections, in particular CMV. We have shown here that allospecific CTL reactivity within a transplanted heart is upregulated during CMV infection, while before the first clinical signs of infection were observed, the cytolytic activity was comparable to that in a control group without infection. The increased donor reactivity of cardiac graft infiltrating CTLs during infection was especially evident in patients who also had acute graft rejection episodes.

This is consistent with reports by others, in which a connection was described between active CMV infection and acute rejection or graft-versus-host disease in transplant recipients (1-6). Another illustration of the mutual relation between infection and alloreactive responses is the finding of Cray and coworkers (31), who demonstrated in mice that inoculation of CMV augmented the effect of intravenous injection of allogeneic immunocompetent cells, resulting in signs characteristic of graft-versus-host disease.

Gaston et al. (7) proposed that virus specific, self-MHC restricted CTL can mediate rejection of an allograft because of their cross-reactive recognition of allogeneic MHC determinants. The data of Yang and collegues (8,9) do not exclude this possibility, but suggest that this does not account for the bulk of the allospecific killing observed during acute virus infections: cold inhibition studies showed that the virus specific and the allospecific cytotoxic T cells were separate populations.

Our finding of increased class I directed cytotoxicity during CMV infection is in agreement with findings of others, who showed that the virus could directly enhance the expression of MHC class I antigens and of adhesion molecules (11,12). The combination of virus-induced generation of

allospecific CTL and upregulation of MHC antigens and adhesion molecules on graft tissue may result in rejection. This rejection process may in turn amplify the virus infection by upregulating HCMV-attachment receptors, which could be MHC class I molecules according to Grundy (11,13) or specific HCMV receptors (32 and 34 kDa cell surface glycoproteins) according to Nowlin (14).

This amplification of one process by the other may account for our finding that the increased class I directed cytotoxicity was most evident in patients with both infection and rejection, although there was individual diversity within the patient groups. This may be caused by genetic variability among individuals in the susceptibility to the virus, as was shown in mice by Grundy and associates (13), or to genetic factors that determine whether the patient is a high or a low responder to foreign HLA antigens (32,33).

Both CMV infection and acute rejection can act as initiating factors of this amplification process. Alternatively, anti-rejection therapy, which suppresses CMV specific CTL responses, may result in prolonged viraemia (1,2,3,15,16). This could in turn cause a prolonged alloreactive cytotoxic response.

In patients with primary CMV infection, not only class I, but also class II directed cytotoxicity of graft infiltrating cells was found to be increased during infection. Among these patients a high incidence of CMV disease was observed, which is a well known complication in CMV seronegative recipients of organs from seropositive donors (2,19,34,35). Von Willebrand et al. (36) found an increased expression of HLA class II antigens on graft structures in patients with disseminated CMV infection, which was presumably induced by lymphokines produced by the abundantly present virus-activated mononuclear cells. The increased class II expression possibly plays a role in the initiation of a primary immune response against CMV, which involves interaction between class II positive cells presenting viral antigens to T helper cells in the context of the class II molecule. The high level of class II antigen expression in the graft during primary infection can also attract class II specific CTLs. In contrast, a secondary response against CMV will predominantly consist of primed class I reactive CTL.

In conclusion, although there is already a rather extensive literature on the association between CMV infection and clinical graft rejection, only few data are available regarding the influence of the virus infection on the cytotoxic T cell response within a human allograft. We have now shown evidence that allospecific cytotoxic T cell reactivity within a transplanted heart is upregulated during CMV infection.

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SUMMARY

Even under adequate immunosuppression, organ allografts are usually infiltrated by recipient mononuclear cells that often display alloreactivity in in vitro assays. The purpose of the studies presented in this thesis was to investigate the phenotypic composition and allospecificity of endomyocardial biopsy derived T cell lines in relation to several parameters, such as acute rejection, the degree of HLA matching, the immunosuppressive therapy used, and infection with cytomegalovirus, a common pathogen in transplant recipients.

In chapter 1 an overview is given on several aspects of clinical heart transplantation. Rejection is still a major cause of graft dysfunction and failure, and results from cellular and humoral responses against foreign antigens. Of the foreign antigens that may be recognized by the immune system, the human major histocompatibility complex (MHC) is the most important. Recent literature is reviewed on the structure of MHC molecules and the interaction of T lymphocytes with these molecules. Interaction of activated T cells with MHC-peptide complexes may result in an alloreactive response, and thus in rejection of an allograft. Several types of rejection are discussed: hyperacute rejection, accelerated acute rejection, acute early rejection, and chronic rejection. Graft infiltrating T lymphocytes can be propagated from allograft tissue samples, using interleukin-2-conditioned culture medium. Chapter 2 describes the patient population studied, the methods used to culture lymphoid infiltrates from endomyocardial biopsies, and the methods used to study their functional and phenotypic characteristics.

In chapter 3 we show that the outgrowth of T lymphocytes from endomyocardial biopsies is related both with histological rejection grade and

time after transplantation the biopsy was taken. Furthermore, the phenotypic composition of the T cell lines changed with increasing rejection grade. Both in rejectors and nonrejectors (patients who did or did not experience acute rejection episodes), approximately 80% of T cell lines demonstrated donor directed cytotoxicity. In rejectors, this reacitivity was directed against a broader spectrum of mismatched HLA antigens than in nonrejectors, which may point to a higher polyclonality of the infiltrates in the hearts of rejecting patients. This predominantly multispecific cytotoxic reactivity pattern changed to a more restricted cytotoxicity in time after transplantation.

The study described in chapter 4 shows that limiting dilution assays in the presence of CD8 monoclonal antibodies can be used to discriminate between high and low-avidity cytotoxic T lymphocytes. To investigate whether the presence of such high-avidity cells in human heart transplants may be predictive for acute rejection, we analyzed their frequency in cultures derived from endomyocardial biopsies in 19 patients, 9 of whom had never experienced acute rejection and 10 who had had one or more rejection episodes. In the rejectors, already before histological signs of rejection (myocyte damage) had developed, significantly higher donor-reactive CTL frequencies were found compared with the nonrejectors. After CD8 inhibition, the difference between rejectors and nonrejectors was even more pronounced, with the highest high-avidity donor-reactive CTL frequencies during rejection. These results indicate that these high-avidity CTL are indeed the effectors of acute graft rejection, and that detection of high frequencies of these cells (more than 1000 per million tested cells) may identify those patients who are high responders to certain foreign HLA antigens, and who are at risk of developing one or more acute rejection episodes.

In chapter 5 we show that the number and nature of HLA mismatches between donor and recipient strongly influence the cellular immune response within the transplanted heart. A higher number of HLA mismatches resulted in a higher incidence of donor-directed cytotoxicity of the biopsy derived T cell lines. CML reactivity against HLA-B and DR antigens was significantly more often found than cytotoxicity against HLA-A, which is consistent with studies on the effect of HLA matching on graft survival in both renal and heart transplantation, reporting a strong influence of HLA-B and DR mismatches on graft survival, in contrast to HLA-A antigens. A higher number of HLA-B and DR mismatches was also associated with a lower freedom from rejection. Of the HLA-A antigens, HLA-A2 appeared to be most immunogenic.

Graft infiltrating lymphocytes from patients who were prophylactically treated with OKT3 or horse antilymphocyte globulin (H-ALG) were found to have different specificity patterns from those in the control group that received cyclosporine (CsA) from the day of transplantation (chapter 6). This

prophylactic treatment led to a significant decrease of the HLA-DR-directed cytotoxicity, while the cytolytic response against HLA-class I mismatches was hardly affected.

It has been shown in the sixties by Monaco and Medawar, that antilymphocytic antisera can abrogate an alloimmune response, but the immune response against an allograft always regenerated after some time. One can speculate that after such treatment, the patient has to acquire a new T cell repertoire in the presence of a transplanted organ. This may result in another composition of the HLA-A, B and DR-directed T cell response than in individuals who received CsA from the day of transplantation. Our data suggest that OKT3 and H-ALG influence the specificity of the T cell allorepertoire, resulting in a decreased frequency of class II-specific cytotoxic T cells after transplantation. H-ALG also has a downregulating influence on the CTL response against HLA class I (HLA-B) antigens. In some patients a fast regeneration of these cells occurs, which results in a higher rejection incidence during the first posttransplant year.

During cytomegalovirus (CMV) infection, both primary and secondary infections, a higher proportion of the EMB derived T cell lines showed cytolytic activity against donor antigens (chapter 7), when compared with T cell lines from patients without infection, in a comparable period after transplantation. This was most evident in patients with both CMV infection and acute rejection episodes when compared with patients with only one of these complications within the first half year after transplantation. This may indicate that both CMV infection and acute rejection can act as initiating factors of an amplification process resulting in an alloreactive reaction. In secondary infections, only an increase of donor class I-directed cytotoxicity was found, while in primary infections cytotoxicity against both donor class I and II antigens were increased.

In conclusion, the studies described in this thesis show that alloreactive lympoid cells can be propagated from endomyocardial biopsies at any time after transplantation, both in patients with and without rejection. The clinical relevance of our experiments is clear from the fact that the in vitro cytotoxic reactivity-pattern of the T cell lines was influenced by time after transplantation the biopsy was taken, by the histological rejection grade as assessed with Billingham's criteria, by the degree of HLA-matching between donor and recipient, prophylactic immunosuppressive therapy, and by intercurrent cytomegalovirus infection. Furthermore, we showed that detection of cytotoxic T cells with high-affinity receptors for donor antigens can identify those patients who are at risk of developing one or more acute rejection episodes.

SAMENVATTING

Zelfs onder adequate immunosuppressieve therapie worden vaak cellulaire infiltraten aangetroffen in orgaantransplantaten. In in vitro studies vertonen deze cellen vaak reactiviteit tegen donor-antigeen. Het doel van de studies beschreven in dit proefschrift was het onderzoeken van de fenotype-samenstelling en de allospecificiteit van T cellijnen verkregen uit endomyocardbiopten uit het harttransplantaat, en deze bevindingen te relateren aan verschillende parameters, zoals acute afstoting, de soort en het aantal HLA mismatches tussen donor en ontvanger, de gebruikte immunosuppressieve therapie, en infectie met cytomegalovirus, een belangrijk pathogeen micro-organisme bij transplantatie patienten.
In hoofdstuk 1 wordt een overzicht gegeven van verschillende aspecten van klinische harttransplantatie. Afstoting is nog steeds een belangrijke oorzaak van stoornissen in de functie van het transplantaat, en is het gevolg van cellulaire en humorale immunologische reacties tegen lichaamsvreemde antigenen. Van de alloantigenen die herkend worden door het immuun-systeem is het menselijke 'major histocompatibiltiy complex' (MHC) het meest belangrijk. Er wordt een overzicht gegeven van recente literatuur over de structuur van MHC-moleculen en de interactie van T lymfocyten met deze moleculen. Interactie van geactiveerde T cellen met MHC-peptide complexen kan resulteren in een alloreactieve respons, en vervolgens in acute afstoting van het transplantaat. Verschillende vormen van afstoting worden geschetst: hyperacute afstoting, versneld acute afstoting, acute afstoting en chronische afstoting.
Transplantaat-infiltrerende lymfocyten kunnen gekweekt worden uit biopten afkomstig van het getransplanteerde orgaan. Hiervoor wordt interleukine-2

bevattend kweekmedium gebruikt. Hoofdstuk 2 beschrijft de bestudeerde patientengroep, de methoden voor het kweken van lymfoïde infiltraten uit endomyocard biopten en de gebruikte testen voor functie- en fenotype-analyse van de cellen.
In hoofdstuk 3 wordt beschreven dat de uitgroei van T lymfocyten uit endomyocardbiopten een positieve relatie vertoont met een hogere histologische rejectie-klassificatie. Ook de fenotype-samenstelling verandert bij een hogere rejectie-graad. Voorts neemt reeds enkele maanden na transplantatie de mogelijkheid T cellijnen te verkrijgen uit de biopten af, en verandert het reactiviteitspatroon van de cellen. Zowel bij afstoters als bij niet-afstoters (patienten die nooit acute afstotingen doormaakten, 25% van de bestudeerde groep), werd in ongeveer 80% van de T cellijnen donor specifieke cytotoxiciteit gevonden. Deze cytotoxiciteit was bij de afstoters tegen een breder spectrum van de gemismatchde HLA-antigenen gericht dan bij de niet-afstoters, wat zou kunnen wijzen op een grotere polyclonaliteit van de infiltraten bij de afstoters. Dit multispecifieke cytotoxiciteitspatroon veranderde in een meer gerestricteerd patroon langer na transplantatie.
De studie beschreven in hoofdstuk 4 laat zien dat met behulp van frequentie-analyse van alloreactieve T cellen met of zonder antilichamen gericht tegen CD8 een onderscheid gemaakt kan worden tussen cytotoxische T lymfocyten (CTL) met een hoge en lage aviditeit voor donor-antigenen. Teneinde te onderzoeken of de aanwezigheid van deze hoog avide cellen in het transplantaat een voorspellende waarde heeft voor het ontwikkelen van acute afstoting, werd frequentie-analyse gedaan op de T cellijnen verkregen uit biopten afkomstig van 19 patienten, 9 niet-afstoters en 10 afstoters. Bij de afstoters kon al in biopten afgenomen voor de eerste histologisch aangetoonde afstoting, significant hogere frequenties van donorreactieve CTL aangetoond worden dan bij de niet-afstoters. Na inhibitie met CD8 monoclonale antilichamen werd dit verschil nog groter. De hoogste frequenties van deze hoog-avide donorreactieve CTL werden gevonden tijdens afstoting. Deze resultaten steunen de hypothese dat deze hoog avide CTL inderdaad de effectoren zijn van acute afstoting, en dat detectie van hoge frequenties van deze cellen (meer dan 1000 per miljoen geteste cellen) die patienten kan identificeren die high responders zijn tegen donor-antigenen, en dus een verhoogd risico hebben voor het ontwikkelen van acute afstoting.
In hoofdstuk 5 wordt beschreven dat HLA mismatches tussen donor en ontvanger een grote invloed hebben op de cellulaire immuunrespons in het getransplanteerde hart. Bij een groter aantal HLA mismatches wordt een hogere incidentie van donorspecifieke cytotoxiciteit gevonden in de T cellijnen verkregen uit endomyocardbiopten. Cytotoxische reactiviteit tegen HLA-B en DR antigenen wordt significant vaker gevonden dan gericht tegen HLA-A antigenen. Dit is stemt overeen met bevindingen in studies waarin

het effect van HLA matching werd beschreven op de transplantaatoverleving in nier- en harttransplantatie. Hierbij werd beschreven dat met name HLA-B en DR mismatches een grote invloed hebben op de transplantaatoverleving, terwijl van HLA-A mismatches geen effect werd gezien. Een hoger aantal HLA-B en DR antigenen is ook geassocieerd met een lagere 'freedom from rejection'. Van de HLA-A antigenen lijkt A2 het meest immunogeen te zijn.
Transplantaatinfiltrerende lymfocyten van patienten die profylactisch behandeld zijn met OKT3 of H-ALG, respectievelijk mono- en polyclonale antilichamen gericht tegen T lymfocyten, blijken een andere cytotoxische specificiteit te vertonen dan die van patienten die direct met cyclosporine werden behandeld na transplantatie. De behandeling met OKT3 en H-ALG resulteerde in een significante afname van de DR-gerichte cytotoxiciteit, terwijl de cytotoxische respons tegen HLA-klasse I donor antigenen nauwelijks beïnvloed werd. In de zestiger jaren hebben Monaco en Medawar reeds laten zien dat antilymfocyten antisera een alloimmuunrespons lam kunnen leggen. Er trad echter na enige tijd steeds een regeratie op van de immuunrespons tegen een allotransplantaat. Men kan speculeren dat na deze behandeling de patient een nieuw immuunrepertoire moet opbouwen in de aanwezigheid van een getransplanteerd orgaan. Dit kan leiden tot een andere samenstelling van de HLA-A, B en DR-gerichte T cel respons dan in individuen die alleen met cyclosporine werden behandeld.
Tijdens cytomegalovirus (CMV) infectie, zowel primaire als secundaire infecties, werd in een groter deel van de T cellijnen cytotoxiciteit tegen donor-antigenen gevonden dan in T cellijnene afkomstig van patienten zonder deze infectie (hoofdstuk 7). Dit was het meest duidelijk bij patienten met zowel CMV infectie als acute afstotingen in het eerste half jaar na transplantatie, in tegenstelling tot patienten met slechts een van deze complicaties in deze periode. Dit kan erop wijzen dat zowel CMV infectie als acute afstoting als initiërende factor kan optreden van een amplificatieproces dat resulteert in een alloreactieve reactie. Tijdens secundaire CMV infecties werd alleen een toename van de donor-klasse I gerichte cytotoxiciteit gevonden, terwijl bij primaire infecties zowel de reactiviteit tegen donor klasse I als klasse II antigenen was toegenomen.
De studies beschreven in dit proefschrift laten zien dat zowel bij afstoters als bij niet-afstoters alloreactieve lymfoïde cellijnen kunnen worden gekweekt uit endomyocardbiopten op willekeurige tijdstippen na transplantatie. De klinische relevantie blijkt uit het feit dat de in vitro reactiviteitspatronen van de T cellijnen beïnvloed worden door de tijd na transplantatie, de histologische klassificatie van het biopt, het aantal HLA mismatches tussen donor en ontvanger, de profylactische immunosuppressieve therapie, en door intercurrente CMV infecties. Voorts hebben we laten zien dat de detectie van T cellen met hoge aviditeit voor donor-antigenen die patienten kan identificeren die een hoog risico hebben afstotingen te krijgen.

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NAWOORD

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 4 augustus 1960 in Leiden. In 1978 behaalde zij het Atheneum diploma aan de Scholengemeenschap Atheneum-HAVO te Katwijk. In hetzelfde jaar werd begonnen met de studie Geneeskunde aan de Rijksuniversiteit te Leiden. Tijdens de studie heeft zij enige maanden onderzoek verricht bij de vakgroep anatomie (titel: 'De oliva inferior en de olivo-cerebellaire projectie in de kip'), onder leiding van Prof. Dr. J. Voogd en Dr. H.K.P. Feirabend. Vervolgens werd meegewerkt aan een onderzoek van Prof. Dr. M.R. Daha in het laboratorium Nierziekten van de Rijksuniversiteit te Leiden, waar de rol van leucocyten en complementfactoren werd onderzocht bij immuuncomplex-depositie onder endotheel. Na het behalen van het artsexamen in 1987 werkte zij enige maanden als keuringsarts bij de Medische Dienst van de Districtsbureau's voor de Arbeidsvoorziening in Zuid- en Noord Holland. Vervolgens werkte zij tot 1992 in het Laboratorium Interne Geneeskunde I in het Thoraxcentrum van het Academisch Ziekenhuis Rotterdam Dijkzigt aan het onderzoek dat ten grondslag ligt aan dit proefschrift (Promotoren: Prof. Dr. W. Weimar en Prof. Dr. E. Bos). Van april 1992 tot januari 1994 was zij werkzaam als arts-assistent op de afdeling Interne Geneeskunde I in het Dijkzigt Ziekenhuis te Rotterdam (hoofd: Prof. Dr. M.A.D.H. Schalekamp). Sinds januari 1994 is zij in opleiding tot internist bij dezelfde afdeling. In het kader van deze opleiding is zij de komende twee jaar werkzaam op de afdeling Interne Geneeskunde in het Reinier de Graaf Gasthuis te Delft (opleider: Dr. W. Hart).