INTRAGRAFT CYTOKINE mRNA EXPRESSION AFTER CLINICAL ORGAN TRANSPLANTATION

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE ERASMUS UNIVERSITEIT ROTTERDAM OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR P.W.C. AKKERMANS M.A.

EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP WOENSDAG 20 MEI 1998 OM 15.45 UUR

DOOR

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GEBOREN TE GOUDA

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ISBN 90-9011463-7

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Intragraft cytokine mRNA expression after clinical organ transplantation

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This thesis was printed by Haveka B.V., Alblasserdam.

CONTENTS

Chapter 1

INTRAGRAFT CYTOKINE GENE EXPRESSION: IMPLICATIONS FOR CLINICAL TRANSPLANTATION (overview)

This chapter is based on the article published in:

Transplant International (in press)

Carla C. Baan and Willem Weimar

ABSTRACT

As the knowledge of the cytokine network in experimental transplant models grows, we need to understand how and to what extent cytokines mediate the various donor-directed immune events in clinical situations. This overview on clinical cytokine measurements shows that specific intragraft cytokine messengerRNA (mRNA) expression profiles can be associated with acute rejection, may reflect the efficacy of immunosuppression, and can trace patients at risk for the development of early chronic rejection. Moreover, the literature also showed that acute rejection and immunological quiescence in man are not restricted to the cytokine patterns according to the type 1/type 2 paradigm. This apparent lack of association may be caused by the immunosuppression used in the clinic but may also be the result of the infinite diversity of donor and recipient factors of which polymorphisms in cytokines and cytokine receptor genes may play a central role.

INTRODUCTION

Studies in animal transplant models have clearly proven the value of cytokine measurements. These studies learned us a lot about the cytokine network during the full spectrum of transplant associated immune responses. However, it is important to understand to what extent these mostly clear findings can be translated to the clinic and whether alterations in the cytokine pattern may help the clinician to adapt the immunosuppressive regimen in a given individual.

Measurements of cytokines became very popular with the introduction of the reverse transcriptase-polymerase chain reaction (RT-PCR) and the availability of enzyme linked immunosorbent assay (ELISA) kits in the early 90's. These techniques made it possible to monitor both cytokine messenger RNA (mRNA) expression in tissues and cytokine protein concentrations in body fluids. *In situ* hybridization, bioassays, and immunohistochemistry were also applied to determine the presence of cytokines. All these methods resulted in a tremendous amount of articles on cytokine measurements analyzing various stages of the immune response after transplantation. Indeed studies in experimental animal transplant settings showed

that cytokines played key roles at each of these stages. However, in contrast to the clearcut data derived from studies under strict controlled laboratory conditions the results in clinical transplant settings proved to be significantly more difficult to interpret. From large database studies it is already known that graft rejection and patient survival are influenced by infinite diversity of donor and recipient factors, including age and accompanying diseases of organ donor and recipient, former blood transfusions, HLA compatibility match grade, and history of viral infections. Moreover, in clinical transplantation nowadays a variety of immunosuppressive schedules can be used that can affect various steps in the cascade of immune activation, albeit not always to the same degree in each patient. Consequently, the variability between individual patients will affect the results of cytokine measurements. Most of the data relating local cytokine production with immune mechanisms after clinical transplantation is based on *in situ* detection of transcriptional factors by RT-PCR. This technique is suitable to measure a broad panel of cytokine mRNAs in small amounts of tissue such as biopsies. These cytokine measurements may provide information on immune events (acute and chronic rejection and graft acceptance) in individual patients. This review, summarizes these intragraft cytokine measurements after heart, liver and kidney transplantation and will discuss the typical pitfalls associated with patient directed research which may influence the outcome of intragraft cytokine mRNA measurements.

CHARACTERISTICS OF CYTOKINES

Cytokines are soluble polypeptides which function as a paracrine or autocrine mediator acting over short distances regulating a variety of immune and inflammatory responses. Since the discovery of the T cell growth factor, presently known as IL-2, in 1978, the field of soluble mediators has been explosively explored (30). A complete network of interleukins, interferons, chemokines and growth factors has been characterized. Cytokines secreted under normal or pathological conditions affect proliferation, differentiation, and the function of cells involved in numerous

physiological processes. Their biological activity is mediated by specific membrane receptors which can be expressed on a variety all cell types. Cytokines can have stimulatory and inhibitory properties, may synergise or antagonize the action of individual components in the network. Additionally, one factor may replace or compensate for the lack of another cytokine (redundancy) but in the context of a particle immune function e.g. graft rejection individual cytokines may have a dominating role. In the field of organ transplantation this may hold especially true for T cytokines. In 1991 Romagnani showed that human T helper (Th) cells like mouse CD4+ T cells express functionally distinct cytokine profiles (57,73). The Th1/Th2 paradigm was born. According to this theory, Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-β and favour cellular immune responses, delayed type of hypersensitivity, macrophage activation, while Th2 cells secrete IL-4, IL-5, IL-6, IL-10 favour tolerance, and stimulate B cell differentiation and antibody responses (62). Recently, it has become evident that this dichotomy is not confined to CD4 helper T cells alone (58). CD8 effector cells and γδ T cells may also secrete cytokines in a polarized fashion. This has led to the more generalized nomenclature: type 1 and type 2 cytokines (18).

CYTOKINE MEASUREMENTS IN EXPERIMENTAL TRANSPLANT SETTINGS

Studies in experimental transplant settings had shown that immune responses towards an organ are regulated by cytokine interactions (24,36). From these and other studies, we learned that endothelium damage by ischemia, reperfusion and surgery triggers non-specific inflammatory responses mediated by members of the different cytokine families (24,31,36,38,51). Damaged endothelial cells release increased amounts of the IL-1ß and IL-6, IFN-γ, the chemokines macrophage chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and MIP-1ß and IL-8, colony stimulating factors, and multiple growth factors such as TNF-α, platelet derived growth factors (PDGF), insulin growth factor-1, transforming growth factor (TGF)-ß, basic fibroblast growth factor (FGF) (4,25,70,76). Thus, as a result of the surgical procedure a complete network of cytokines is already activated,

even before allogeneic reactions can be encountered. Cytokine production by activated endothelium results in upregulated HLA expression and increased adherence of monocytes and T cells which is followed by infiltration into surrounding tissue. For instance, TNF-α and IL-1ß induce vascular endothelial cells to transcribe the vascular adhesion molecule-1 (50). Consequently, the triggering step is crucial because non-specific endothelium injury can be the initial factor in the development of acute and chronic graft rejection.

The first experimental studies analyzing molecular pathways involved in acute rejection and graft acceptance showed that these mechanisms are dominated by intragraft production of either type 1 cytokines or type 2 cytokines, respectively. In rodents and cynomolgus monkeys, acute rejection was accompanied by intragraft type 1 (IL-2, IFN-γ) mRNA expression, suggesting that these cytokines control the rejection process after kidney, heart and liver transplantation (24,56,89,101). Studies in experimental models also showed that graft acceptance can be identified on basis of a clear intragraft mRNA profile. In the majority of these studies, graft acceptance is associated by diminished type 1 cytokine and enhanced type 2 cytokine production (16,19,77,88). However, it is evident from studies using genetically manipulated animals that type 1 cytokines are not always required for rejection and that type 2 cytokines do not always initiate permanent engraftment. IL-2 and IFN-γ knockout mice reject their transplants in the presence of type 2 cytokines (45,85) and IL-4 knockout mice accept their graft in the presence of type 1 cytokines (59). These studies indicate that immunological phenomena such as graft rejection and acceptance are not exclusively restricted to the type1/type 2 dichotomy. Alternative and/or redundant pathways may contribute to the alloimmune response associated with these phenomena. Interestingly, results from the double IL-2 and IL-4 knockout model suggested that also T cell growth factors produced by non T cells are able to induce allograft rejection (74).

Evidence for a significant role of cytokines in the etiology of chronic allograft rejection or transplant arteriosclerosis has been found in experimental transplant studies as well. These studies are focused on cytokine production by injured endothelial cells and smooth muscle cells and on chemokine production by activated

macrophages. Semi-quantitative RT-PCR analysis in rat aortic allografts showed that mRNA expression of various cytokines (e.g. IL-1ß, TGF-ß, PDGF, TNF-α, insulin growth factor-1, acid FGF, basic FGF) are upregulated (38,70). Chemokines produced by the infiltrated mononuclear cells may also play a critical role in chronic rejection processes. In the Lewis to F344 cardiac rat model, mRNA expression of the chemokines MCP-1, allograft inflammatory factor-1, allograft inflammatory factor-2 is elevated (76,92). In this model, transplant arteriosclerosis is also associated by intragraft IFN-γ and IL-6 mRNA expression located in the infiltrating cells.

Thus, analysis in animal transplant settings showed that cytokines play key roles at every stage of the immune response after transplantation. Especially the association between acute cellular rejection and production of type 1 cytokines within the graft prompted many investigators to analyse the role of these cytokines after clinical kidney, liver and heart transplantation.

T-CELL ACTIVATION AND THE EFFECT OF IMMUNOSUPPRESSIVE AGENTS ON CYTOKINE PRODUCTION

The immune response after transplantation is largely governed by actions of T cells. Initiation of T cell responses requires interaction of the T cell receptor with processed donor antigen, binding of CD4 and CD8 proteins with HLA class II and class I molecules, and secondary costimulatory signals between ligands on the T-cell (CD28, CD40Ligand, CD2, LFA-1) and their counterparts (B7/CTLA-4, CD40, LFA-3, ICAM) present on antigen presenting cells including dendritic cells, Mϕ, B-cells and T-cells (figure 1; 42,49,79,82). In addition to cell surface events soluble mediators (IL-1, IL-6) also contribute to T cell activation (78). The interactions trigger a number of intracellular events leading to production of cytokines and their receptors and cellular proliferation. These intracellular events involve the activation of tyrosine kinases, tyrosine phosphorylation of cellular proteins followed by elevated intracellular free calcium concentrations and activation of the calcium- and phospholipid dependent protein kinase C (14,75). This cascade leads to the initiation of transcription of cytokines and receptors.

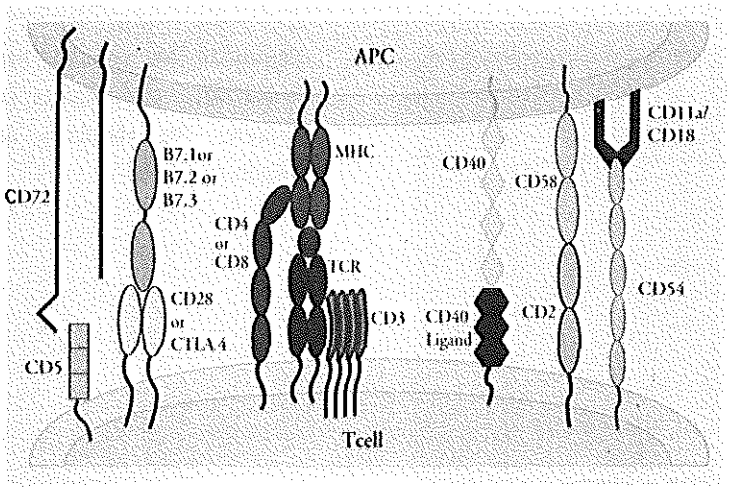

Figure 1. Interaction between antigen presenting cells (APC) and T-cells (adapted from Dallman, The Handbook of Transplant Immunology 1995)

Transcription of cytokine genes is regulated by the binding of regulatory proteins (NF-AT, AP-1, OCT) to specific DNA sequences in the enhancer region of the gene (72). The immunosuppressive action of cyclosporin A (CsA) and tacrolimus/FK506 is based on inhibition of the T cell signal transduction pathways (figure 2). In activated T cells, the rise of calcium activates the calmodulin-dependent phosphatase calcineurin. CsA and tacrolimus/FK506 inhibit calcineurin activity when it forms a complex with immunophilins resulting in reduced cytokine production (33,40). In contrast to activation signals provided through the T cell receptor, the effects of the CD28 costimulatory signals are not inhibited by CsA and tacrolimus/FK506 (99). Glucocorticosteroids inhibit cytokine gene expression at multiple sites of the activation cascade. Steroids blocks cytokine production of T cells by inhibiting the IL-1 and IL-6 production of antigen presenting cells, by inhibiting calcineurin-dependent pathways (65), and by interfering with the binding and/or transcriptional activity of NF-AT, OCT, AP-1 of the IL-2 gene (65,93). In contrast, both rapamycin, although structurally very similar to tacrolimus/FK506, and mycophenolate mofetil, have no effect on cytokine mRNA transcription (103). Rapamycin blocks cytokine driven T

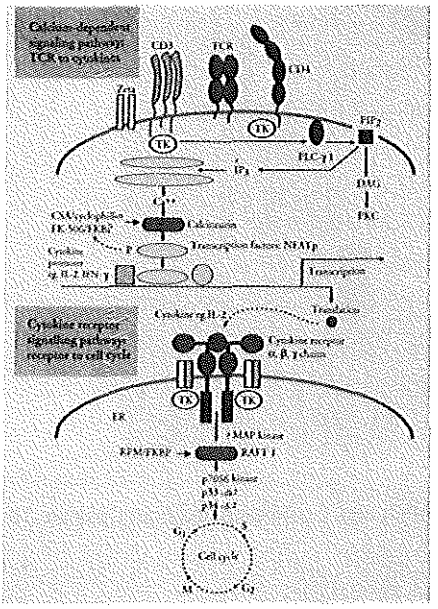

Figure 2. The molecular mechanisms of immunosuppressive agents (adapted from Halloran, The Handbook of Transplant Immunology 1995)

cell proliferation by affecting proteins that are involved in cell cycle pathway and mycophenolate mofetil blocks the purine *de novo* synthesis which is required for DNA synthesis (15). Another approach to inhibit allogeneic immune responses is to block T-cell co-stimulation. Blockade of the CD28/B7 and/or CD40/CD40Ligand interactions results in prolonged survival of allografts which can be associated by shifts in the type 1/type 2 balance (49,68,77). Since cytokines appear to play major roles in transplant pathology, their receptors may also be good target for selective immune therapy strategies. In view of the importance of IL-2 in the rejection process, monoclonal antibodies were developed to block the IL-2 dependent signalling pathway (44,60,84,96,97). The high affinity IL-2Receptor (IL-2R) consists of three transmembrane protein chain termed α, ß, γ. The α-chain is not expressed on resting T cells, but is induced following activation. Moreover, the α-chain is necessary for the formation of the signal transducing high affinity receptor (43). Significant signal transduction components of cytokine receptors are members of the Janus-family kinases (JAKs) and signal transducers and activators of transcription (STATs). Cytokines may use common JAKs and STATs which may at least in part

explain phenomena of cytokine pleiotropy and redundancy (43). For example the γ-chain or the common γ_c subunit of the IL-2R is a component of high affinity receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 while the overlapping biological properties of IL-4 and IL-13 may be explained by an another common receptor (17). Thus, blockade of cytokine receptors by monoclonal antibodies is a method to inhibit cytokine driven signal transduction pathways.

Interference at any level of the T cell activation cascade by immunosuppressive agents is associated by changes in cytokine mRNA expression within the allograft. Therefore, monitoring of intragraft cytokine mRNA expression by RT-PCR is an ideal tool to determine the immune-status of graft infiltrating cells. This type of analysis may be helpful to treat patients more specifically with immunosuppressive therapies. However, extrapolation of *in vitro* or experimental data to the clinic requires care. Interpretation of cytokine measurements may be hindered by all kinds of patient related complications.

INTRAGRAFT CYTOKINE MEASUREMENTS AFTER CLINICAL ORGAN TRANSPLANTATION:

acute rejection

In transplantation immunology, shifts in the cytokine balance are often used to study mechanisms regulating acute rejection (type 1 response) or down-regulation of the immune response (type 2 response). Consequently, most of the cytokine measurements in the clinical field were also focused on mediators produced by activated infiltrating T lymphocytes (table 1). In clinical kidney transplantation, the first prove of a predominance of type 1 cytokines during early stages of allograft rejection was reported by Dallman et al. (23). In that report, intragraft IL-2 mRNA expression measured in fine needle aspirates preceded clinical rejection. Another study using fine needle aspirates also showed an association between intragraft type 1 (IFN-γ) cytokine mRNA expression and clinical acute rejection (61). In contrast, Krams et al. did not find a relation between IL-2 positivity and different stages of rejection (46). They only occasionally found message coding for the IL-2

gene in solid biopsies from rejecting or rejected kidneys. The group of Hutchinson was also not able to confirm an association between intragraft IFN-γ mRNA expression and kidney graft rejection (41). Conflicting data on the involvement of type 1 were reported from studies using *in situ* hybridization too. Studies by Vandenbroeke et al. (98) and Grimm et al. (35) showed that neither IL-2 nor IFN-γ mRNA expression was associated with acute kidney allograft rejection, whereas Loong and colleagues found enhanced cytokine protein production of both type 1 (IL-2, TNF-α), their receptors (IL-2R), and type 2 (IL-4, IL-6, IL-10) cytokines in rejecting kidneys (52). In line with this observation is the study by Xu et al (102). In this study, intragraft expression of both type 1 (IL-2) and type 2 (IL-10) cytokines was upregulated during renal allograft rejection. Such a prominent role for type 2 cytokine in the rejection process after kidney transplantation was published by several groups. Krams et al (46) found in rejection biopsies and rejected kidneys IL-4 and IL-5 mRNA expression whereas Strehlau et al. (86) found heightened IL-10 mRNA expression in the absence of IL-4 mRNA.

As in kidney transplantation controversial data on monitoring type 1 cytokines during rejection are reported in clinical liver and heart transplantation. Indeed, several investigators demonstrated that type 1 cytokines were detected or upregulated in rejecting livers (8,13,20,29,32), and hearts (9,22,34) but these and other studies also demonstrated that both hepatic and cardiac allograft rejection may occur in the absence of type 1 cytokines (48,53,105). Thus, unlike the situation in animal models, the role of type 1 cytokines in the acute rejection process after clinical transplantation is less clear. From clinical studies it is obvious that allograft rejection may not only occur in the presence of both type 1 and type 2 cytokines but also in their absence. However, the presence of type 1 cytokines is nearly always associated with rejection and is downregulated again after successful antirejection treatment (7,39). Absence of IL-2 mRNA expression can be found during immunological quiescence, but may also simply indicate that the IL-2 mRNA signal has vanished already during the rejection process. IL-2 is an early gene in the course of an immune response and Dallman did show that IL-2 mRNA expression preceded the clinical rejection (21,23). Moreover, kinetics of IL-2 mRNA expression

showed that IL-2 mRNA is only briefly expressed by graft infiltrating lymphocytes. After stimulation with donor antigen, IL-2 mRNA expression was detectable as early as 1-2 h, reached maximum levels between 2-48 h, while base-line levels were again approached by 20-72 h (11). Accordingly, timing is a factor which significantly complicates cytokine measurements. The IL-2 mRNA signal could have been easily missed. Another explanation for IL-2 negative rejection is redundancy in the cytokine network. Recently, we found that proven blockade of the IL-2/IL-2R signalling pathway by CsA and an anti-IL-2R monoclonal antibody is not sufficient to prevent allograft rejection in cardiac allograft recipients (96). Other cytokines may adopt the function of IL-2. For instance, intragraft IL-7 and IL-15 (a T cell growth factor secreted by macrophages) mRNA expression are present in the IL-2 negative renal and liver allograft rejections and may well serve as initiators of the allogeneic process (6,67,86).

chronic rejection

Chronic allograft rejection is characterized by ongoing inflammation and diffuse concentric intimal proliferation in the arterial system (12,83). This chronic process is thought to derive from an interaction between non-specific and allogeneic factors, leading to smooth cell proliferation. The role of cytokines in the pathogenesis of transplant arteriosclerosis has been recognized for many years. The relationship between ischemia/reperfusion injury and development of chronic rejection is well-known although the underlying mechanism is poorly defined. Recently, Adams et al. showed that perfusion/ischemia leads to secretion of the chemokines macrophage inflammatory protein-1α and macrophage inflammatory factor-1ß by endothelial cells of the transplanted liver (1). Chemokines produced by activated T cells and macrophages may also mediate the development of arteriosclerotic lesions. In comparison to non-transplanted controls, the chemokine RANTES (regulated on activation normal T cell expressed and secreted) mRNA and protein production has been shown to be upregulated in coronary arteries obtained from patients with endstage chronic rejection after clinical heart transplantation (66). Using *in situ* hybridization and immunohistochemistry, RANTES was localized in the infiltrating

mononuclear cells and endothelial cells. In vitro studies showed that RANTES is produced by endothelial cells after exposure to TNF-α, IL-1β, or IFN-γ (27). Therefore, routine intragraft chemokine measurements of time-zero -and the first posttransplant biopsies may provide insight into how early chemokine production by endothelial cells or macrophages mediate mechanisms leading to acute and chronic rejection.

Apart from ischemia-reperfusion injury, the typical non-specific factors, specific immune responses to the allograft can induce chronic inflammatory processes. This is based on the observation that a high incidence of acute rejection episodes is associated with the occurrence of chronic rejection. T cell derived cytokines such as IFN-γ may not only mediate the acute allograft response but increase the expression of HLA and adhesion molecules and stimulate smooth muscle cell proliferation. However, evidence that graft arteriosclerosis is mediated by type 1 or type 2 cytokines is limited (table 2). We found that production of type 1 cytokines preceded the diagnosis of chronic rejection after heart transplantation. Intragraft IL-2 mRNA expression during the first acute rejection episode and IFN-γ production by graft infiltrating lymphocytes in the first 6 months posttransplant was associated with early development of chronic rejection (10,94). In long-term transplanted grafts endomyocardial lymphoid infiltration is common but these cells do not transcribe detectable IL-2 nor IL-4 mRNA, although mRNA expression of various growth factors was present (10). This observation suggests that cells producing type 1 cytokines are mainly involved in the initiation and not in the maintenance of transplant arteriosclerosis after heart transplantation. In liver grafts an association between type 1 cytokines and end-stage chronic rejection was reported by Hayashi et al (37). However, intragraft analysis in renal biopsies showed that not type 1 cytokines are involved but that type 2 cytokines are associated with graft arteriosclerosis (52,55,64). These studies show that T cells producing type 1 and type 2 cytokines are both involved in the pathogenesis of chronic rejection. PDGF, basicFGF, insulin growth factor-1, TNF-α, TGF-β, are well characterized growth regulators for endothelial cells and smooth muscle cells. An association between chronic rejection and the presence of one or more of the above mentioned growth factors in the graft

was reported by several groups (table 2). Messenger RNA expression of acidicFGF and basicFGF is upregulated in most of the transplanted hearts, irrespective of their chronic rejection state, implying that these mediators play a role in processes after transplantation but their specific involvement in the development of transplant arteriosclerosis still is not totally elucidated (3,105). The role of TGF-β, TNF-α, and PDGF in chronic rejection appears more clear. In biopsies and specimens from grafts of patients who died from graft failure due to chronic rejection the expression of TGF-β, TNF-α, PDGF was related to this chronic complications (2,26,37,52,80,81,104). However, interpretation of most of these data is limited, findings were compared to non-transplanted tissue instead of transplanted organs without signs of chronic rejection. Moreover, cytokine production may be affected by the hypotensive period with warm ischemia during the terminal phase of life. Despite these remarks, it is assumable that increased production of TGF-β, TNF-α, PDGF may be of importance in the mediation of growth and repair mechanisms of smooth muscle cells and injured endothelial cells.

CYTOKINES AND GRAFT ACCEPTANCE

Tolerance by donor specific blood transfusion, a brief course of CsA, anti-CD4 mAb pretreatment, blockade of co-stimulatory signals, is often associated by diminished type 1 cytokines and enhanced type 2 cytokines (16,19,77,88). These findings has led to hope that induction of a type 2 response to antigen might achieve donor-specific tolerance. But the redundant and pleiotropic nature of the cytokine network suggests that induction and maintenance of transplant tolerance depends on complex mechanisms and can not entirely explained by the dichotomy into type 1/type 2 (69). Recently Strom et al. published "the traffic light" hypothesis for tolerance (87). These authors postulate that tolerance induction can take place in the presence of autocrine IL-2 and IL-4 (green light), while in the presence of paracrine IL-2, IL-7 and IL-15 induction can not be established (red light). Thus not the nature of cytokines as an answer to tolerance but the hierarchy of T cell growth factors and their ability to mediate tolerance and rejection determines in their view

the result of cytokine production. After clinical transplantation, IL-4 mRNA expression is frequently measured during histopathologic rejection and occasionally during immunological quiescence (table 1 and table 3; 8,9,20,29,32,46,53). An indication that IL-4 may down-regulate the immune response in patients was recently published. We found that spontaneously resolving liver graft rejection is associated with intragraft IL-4 mRNA expression in the absence of IL-2 mRNA (8). In line with this observation are the data published by Gorczynski and colleagues (32). In a high proportion of liver biopsies obtained from patients without clinical evidence of rejection IL-4 mRNA expression was present in the absence of IL-2 mRNA expression. After kidney transplantation, Kusaka et al. showed that peripheral blood cells from a patient who discontinued all immunosuppressive drugs produced high amounts of IL-4 (47). IL-10 is supposed to down-regulate the donor-specific immune response too. However, only occasionally intragraft IL-10 mRNA expression was associated with immunological quiescence. IL-10 mRNA was present in hearts and livers with stable graft function and absent in rejecting grafts (20,34). However, most of the *in vivo* studies analyzing intragraft IL-10 mRNA expression did not find any evidence for an immunosuppressive function. Instead, a positive correlatation with acute rejection was observed in many cases (5,86,95,102). The presence of type 2 cytokines during acute rejection within the graft can mean two things. They are also involved in the acute allograft response or, which is more interesting, they may create an environment of immunologic non-responsiveness to the allograft. In this view, production of type 2 cytokines may be the response of the immune system to type 1 cytokine induced inflammatory responses, thereby restoring the balance of the cytokine network. In order to further elucidate the role of type 2 cytokines in the clinical transplant setting further controlled studies need to be done. Especially analysis in serial biopsies of IL-4 and IL-10 mRNA expression may provide information about the role of cells producing type 2 cytokines as down-regulators of specific immune responses. Another interesting feature for intragraft type 2 cytokine measurements are its applications to monitor immunosuppression weaning protocols. From such trials much can be learned about the role of type 2 cytokines regarding active mechanisms involved in acceptance of an allograft.

DISCUSSION

The current literature on cytokine production in clinical transplant settings is complex. It shows no consensus about the type 1/type 2 (Th1/Th2) paradigm in transplantation (tables). This is not entirely surprisingly given that data are generated from divers clinical situations. Besides, methodologically, most of the studies differ from another which makes not always possible to compare data. The lack of association in the type 1/type 2 paradigm in allograft responses in man is likely caused by the tremendous heterogeneity between allograft recipients and the complexity of the cytokine network. Most of the biopsies after liver and kidney transplantation are taken in case of deteriorating graft function whereas after heart transplantation biopsies are obtained at a routine basis. Undeniably, definitions in diagnosis have vast consequences on the interpretation of data concerning intragraft cytokine measurements. Accordingly, it is difficult to find clear differentiating cytokines in processes like rejection and graft acceptance. However, these typically clinical complications do not rule out the possibility that type 1 cytokines, IL-2 and IFN-γ, control the alloresponse to solid organ allografts and that type 2 cytokines, IL-4 and IL-10, mediate responses committed with immunological quiescence or even tolerance. Nevertheless, it has become evident that the type 1/type 2 paradigm is an oversimplified model which represent extremes of many possible outcomes (63). It was found that clones, at the same time, can make both type 1 and type 2 cytokines (58). Therefore, we assume that graft infiltrating cells but also cells from the graft itself can produce type 1 and type 2 cytokines during an immune response. Nowadays we are aware of pleiotropism and redundancy of the cytokine network. In IL-2 negative rejections, potent cyclosporin resistant T cell growth factors such as IL-7 and IL-15 mRNA are present which suggests that these mediators participate in the rejection process (6,13,86). Redundancy of the cytokine network also implies that elimination of a single mediator of the cytokine cascade may not be enough to inhibit an allogeneic response. In addition, clinical data can only be interpretated in the context of the specific immunosuppressive drugs used as those agents may interfere with local cytokine production. Several reports suggest that agents that block the calcineurin pathway selectively inhibit type 1 cytokines while sparing type 2

cytokine production (28,71). This may have a great clinical implications. However, there are no clinical studies available supporting these data. The association between type 1 cytokines and acute rejection is often based on timing. In both animal and human studies intragraft IL-2 mRNA expression may precede the rejection episode (23,24,54). However, this early T cell activation marker is not specifically associated with clinically significant rejection. Type 1 cytokines are measured in early serial fine needle aspirates obtained from rejecting as well as from non-rejecting infiltrates probably reflecting a common inflammatory responses due to surgical trauma, ischemia and reperfusion injury (54). Moreover, presence of a particular cytokine at the graft site is not direct evidence of its participation in the rejection response. The fact that local production of type 1 cytokines alone is not sufficient for the development of acute rejection may suggest that not all elements are available for an effective immune response. Unknown cytokines or other components of the immune system may be involved. Even cytokines produced by the graft itself may control the specific immune response.

For chronic rejection the situation is even more complicated. The etiology of chronic rejection is multifactorial and can be distinguished into immunological and non-immunological factors. For instance, oxidatively modified low-density lipoproteins may stimulate secretion of MCP-1 and colony stimulating factors by cultured endothelial cells resulting in enhanced monocyte adherence to these cells (50). This non-specific trigger of cytokine production may also have consequences for acute allogeneic responses, thereby accelerating the development of transplant arteriosclerosis. Despite the overwhelming literature on cytokine measurements in clinical transplant settings, almost none of the research groups has analyzed cytokine mRNA expression in biopsies taken 5-10 years after transplantation. This type of analysis may enlighten some of the mechanisms involved not only in chronic allograft rejection but also in graft acceptance.

Recently, a new approach using PCR applications for cytokine analysis was reported by the group of Hutchinson. They correlated presence of polymorphic microsatellite markers in the TNF-α gene with cytokine production *in vitro* (90). It was found that heart transplant recipients with the microsatellite TNFd3 produced

significantly more TNF-α than TNFd3 negative patients. A mutation at position -308 in the promotor region of the TNF-α gene is also associated with increased TNF-α production while presence of an A at position -1082 in the IL-10 gene is correlated with decreased IL-10 production (91,100). Analysis of these cytokine genotypes showed that heart transplant recipients typed as high TNF-α and low IL-10 producers had significantly more severe acute rejection episodes (91). Fascinating from this type of analysis is the possibility to use cytokine genotypes as a marker to identify patients at risk for rejection or who will accept their allograft already pre-transplant. In this perspective, both donor and recipient genotype are important.

CONCLUSIONS

This review shows that data on the cytokine network gained in experimental studies are, at the most, only an indication for these processes in transplant patients. The many reports on cytokine measurements in patients have resulted in a tremendous heterogeneity of data. Nevertheless, it is possible to characterise specific cytokine patterns within the graft during, acute and chronic rejection and graft acceptance. Although, cytokine profiles may vary between heart, kidney and liver allograft recipients, it was shown that cytokine measurements are extremely useful to monitor the efficacy of immunosuppressive drugs and to characterise changes in cytokine profiles in individual patients. Such an approach may point out specific patients who require more or less or no immunosuppressive therapy. Especially the link between cytokine polymorphisms and cytokine production, a new and promising approach, could be the opening in prospective immunological typing in clinical transplantation.
Until the mechanisms responsible for control of cytokine biologic activities are clarified, we have to be cautious concerning the clinical significance of intragraft cytokine gene expression in transplantation. To elucidate the precise function of cytokines in patients further controlled studies are needed. Monitoring of therapeutic and cytokine intervention trials will help us to unravel the complex cytokine network which eventualy should lead to better care and treatment of transplant patients.

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Table 1: Intragraft cytokine mRNA profiles by RT-PCR associated with **acute rejection** after clinical organ transplantation

organ	cytokine profile associated	cytokine profile not associated	refs	comments
K	IL-2		23	IL-2 mRNA expression preceded clinical rejection
K	IFN-γ		61	IFN-γ mRNA expression preceded clinical rejection
K	IL-4, IL-5, IL-6, TNF-α	IL-2, IFN-γ, IL-7	46	no comparison against either normal kidney tissue or biopsies without signs of rejection was made
K	IL-2 TNF-α, IL-6	IFN-γ, IL-4, IL-6, IL-10, TNF-α IL-2, IFN-γ, IL-4, IL-6	41	early rejections (< day 100 pTX) late rejections (> day 100 pTX)
K	IL-6	IFN-γ	98	technique used: *in situ* hybridization
K	IL-2, IL-10	IFN-γ, IL-4, IL-7, TGF-β	102	compared to chronic allograft nephropathy samples
K	IL-7, IL-10, IL-15	IL-2, IFN-γ, IL-4	86	review article
L	IL-2, IFN-γ, IL-6*, IL-1β*		13	compared to "normal" non-transplanted liver tissue, *reduced levels
L	IL-1ß, IL-2, IFN-γ, IL-6,	IL-4, TNF-α	20	IL-2 only in early rejections

L	IL-2, IL-4, IL-5	IL-6, TNF-α	29	IL-5 only in the tracolimus/FK506 group
L	IL-2	IL-4, IL-15	6,8	compared to both biopsies without evidence of rejection or with histological signs of rejection
L	IL-2, IFN-γ	IL-4, IL-6, TGF-β	32	compared to biopsies without evidence of rejection
L	IL-5	IL-2, IL-4, IL-6, IL-1β, TNF-α	53	compared to biopsies without evidence of rejection
H	IL-2	IL-4, IL-10, IL-1β, TNF-α, TNF-β	22	in severe rejection biopsies, requiring anti-rejection therapy
H	IL-2, IL-6,	IL-4, IL-10	7,9,10	in severe rejection biopsies, requiring anti-rejection therapy which inhibited the cytokine mRNA expression
H	IL-2	IFN-γ, IL-4, IL-6, IL-10, TNF-α	34	IL-2 only in early rejections
H	IL-6, TGF-ß	IFN-γ, IL-4, IL-5, IL-1β, TNF-α	105	only a trend for these cytokines was found
H	none	IL-1ß, IL-2, IFN-γ, IL-4, IL-6, IL-10, TNF-α	48	no elevated cytokine mRNA levels were measured during a rejection period within the first 8 weeks posttransplant
H	IL-2, IL-4, IL-5, IL-8, IL-10, TNF-α	IFN-γ, IL-3, IL-6, IL-9, IL-1β, TNF-β	95	technique used: *in situ* hybridization
H	IL-10		5	technique used: *in situ* hybridization

K, kidney; L, liver; H, heart

Table 2: Intragraft cytokine mRNA profiles by RT-PCR associated with **chronic rejection** after clinical organ transplantation

organ	cytokine profile associated	cytokine profile not associated	refs	comments
K	TGF-ß		81	compared to specimens with signs of acute rejection
K	IL-15	IL-2, IL-7	86	review article; only in 2 out of 4 samples
L	IL-1β, IL-10	IL-2, IFN-γ, IL-6	13	decreased cytokine mRNA expression compared to "normal" non-transplanted liver tissue
L	IL-2, IFN-γ, IL-5, PDGF	IL-4, IL-6, IL-8, IL-10, IL-1β, TNF-α	37	compared to stable grafts
L	TGF-ß		26	technique used: *in situ* hybridization compared to "normal" non-transplanted tissue
H	acidic FGF, PDGF		104, 105	compared to "normal" non-transplanted tissue
H	RANTES		66	technique used: *in situ* hybridization compared to "normal" non-transplanted tissue
H	IL-2	IL-4, IL-6 IL-2, IFN- γ, IL-4, IL-6, IL-10, TGF-β, PDGF	10	the characteristics of the first acute rejection are assocated with the diagnosis of chronic rejecton at 1-year at time of diagnosis of chronic rejection at 1-year
H	basic FGF		3	technique used: Northern blotting compared to "normal" non-transplanted tissue

K, kidney; L, liver; H, heart

Table 3. Intragraft cytokine mRNA profiles by RT-PCR associated with **graft acceptance** after clinical organ transplantation

organ	cytokine profile associated	cytokine profile not associated	refs	comments
L	IL-10	IL-2, IFN-γ, IL-4, IL-6, IL-1β, IFN-γ	20	in stable grafts; IL-10 was absent during acute rejection
L	IL-4	IL-2, IL-15	8	in spontaneously resolving rejections
L	IL-4	IL-2, IFN-γ, IL-6, TGF-β	32	in the absence of histological and clinical signs of rejection
H	IL-10	IL-2, IFN-γ, IL-4, IL-6, TNF-α	34	compared to biopsies with severe rejection

K, kidney; L, liver; H, heart

Chapter 2

AIMS OF THIS THESIS

This thesis describes the use of intragraft cytokine mRNA measurements as a tool to unravel immune responses towards transplanted organs.

With respect to histology, most of the immune responses after transplantation are characterized by cellular infiltration. One of the problems with histologic examination of allogeneic tissue is that these infiltrates do not always correlate with a decline in graft function. The immunologic events responsible for these infiltrates and for triggering (or sometimes preventing) organ dysfunction are not understood sufficiently, although they do clearly influence the interpretation of histologic findings. Interactions between immunocompetent cells of the recipient and the transplanted tissue trigger a number of intracellular events that, in cascade fashion, lead to release of cytokines and cytokine receptors. These mediators control the common inflammatory response and consequently also the donor-directed immune response. The intracellular molecular pathways that allow cells to make specific responses can be monitored by measuring cytokine mRNA expression using the reverse transcriptase polymerase chain reaction (RT-PCR) method. We used this technique to determine whether specific cytokine mRNA patterns within the allograft are associated with acute and chronic rejection, with immunological quiescence, or reflect the efficacy of rejection therapy. Analysis of messenger RNA expression of various cytokines and their cytokine receptors by RT-PCR may clarify some of the mechanisms involved in allogeneic immune responses.

In order to correlate intragraft cytokine measurements with immune phenomena, we have to gain insight in the kinetics of cytokine mRNA and protein production by which the immune competent cells control the allogeneic immune response.

Chapter 3: describes such an analysis in which we tried to determine the kinetics of IL-2 and IL-4 mRNA expression and protein production by graft infiltrating lymphocytes and peripheral blood mononuclear cells at the time of acute cardiac allograft rejection.

Rejection in heart transplant recipients is routinely monitored by histological examination of sequentially taken endomyocardial biopsies. This provided us with the opportunity to study the process of rejection at the molecular level.
Chapter 4: describes our attempts to associate intragraft cytokine mRNA patterns with the "gold-standard" for the diagnosis of rejection: histology.

To prevent early allograft rejection after heart transplantation, patients may receive induction therapy with mouse monoclonal antibodies. In view of the importance of cytokines in the rejection process, their receptors may be good targets for immune therapy strategies.
Chapter 5: specifically characterizes the *in vivo* effects of anti-rejection prophylaxis with the anti-IL-2Receptor monoclonal antibody BT563.

Following the diagnosis of rejection, patients are treated with high doses of glucocorticosteroids. We have evaluated whether intragraft cytokine mRNA patterns can be used as a parameter that will predict the efficacy of methylprednisolone anti-rejection therapy and also studied the effect of this therapy on these molecular parameters.
Chapter 6: deals with both these subjects: a study in heart transplant patients treated with steroid anti-rejection therapy is presented in this chapter.

Chronic complications such as graft vascular disease significantly influence the graft and patient survival. Knowledge about the pathogenesis of chronic rejection after heart transplantation is limited. The cause of this complication is probably multi-factorial. Both non-immune and immune factors may trigger the development of graft vascular disease.
Chapter 7: In this chapter we wondered which of non-immune and immune risk factors might trigger, individually or in combination, early development of graft vascular disease in heart transplant patients.

Tolerance of foreign tissue in the absence of immunosuppression is the "holy-grail" in transplantation immunology. Central for this phenomenon is the question: how is tolerance initiated, regulated and maintained? Do cytokines play a role at any level in this process? The so-called self-limited graft rejection seen in liver allografts provided an ideal clinical model to assess these questions.

Chapter 8: In an attempt to answer these questions, we compared intragraft cytokine mRNA expression patterns in liver biopsies without any evidence of rejection, in biopsies with spontaneously resolving rejections, and in biopsies with histological rejection that were accompanied with clinical rejection.

During an immune response, lack of a single cytokine is compensated by other cytokines. No single cytokine is indispensable in immune responses. Redundancy is a phenomenon which complicates the interpretation of cytokine profiles. Since the transcription of some cytokines is blocked by immunosuppressive agents, other cytokines which are resistant for these agents may take over these functions.

Chapter 9: tries to unveil the roles of IL-2 and IL-15 in liver allograft rejection and to find evidence for redundancy phenomena in the cytokine network.

Chapter 10: summarizes all these studies and gives a general discussion on the relevance of intragraft cytokine measurements for the clinic.

Chapter 3

PATTERNS IN DONOR SPECIFIC mRNA AND PROTEIN PRODUCTION OF Th1 AND Th2 CYTOKINES BY GRAFT INFILTRATING LYMPHOCYTES AND PBMC AFTER HEART TRANSPLANTATION

This chapter is based on articles published in:

Transplant International 1996;9(S1):S237-S240

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Transplant Immunology 1997;5:97-103

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ABSTRACT

During cardiac rejection, we studied the kinetics of IL-2 and IL-4 mRNA and subsequent protein production by *in vivo* "primed" graft infiltrating lymphocytes (GIL) and by "naive" peripheral blood mononuclear cells (PBMC), using semi-quantitative RT-PCR and ELISA. For this purpose, these cells were stimulated for 1 to 72 h with donor and control third party antigens. Only stimulation of GIL with donor-specific antigen resulted in early detectable IL-2 and IL-4 mRNA and protein levels. Maximal relative IL-2 mRNA levels were significantly higher than maximal relative IL-4 mRNA levels (10 to 100-fold) in both GIL and PBMC after donor-specific stimulation. This was accompanied by a maximum protein production of 849 pg/ml IL-2 and 16 pg/ml IL-4 by GIL and of 17 pg/ml IL-2 and undetectable IL-4 production by PBMC.

These results suggest that, after stimulation donor-specific "primed" GIL, and not "naive" PBMC, rapidly produce abundant levels of IL-2 and IL-4 at both the transcriptional and protein level.

INTRODUCTION

After clinical heart transplantation, acute rejection usually occurs within the first three months and is characterized by high levels of inflammation in the graft (1-3). The immune mechanisms responsible for cellular allograft rejection are dependent on the activities of primed allospecific T lymphocytes. Interaction of these immunocompetent $CD4^+$ T helper cells (Th) and $CD8^+$ cytotoxic T lymphocytes (Tc) with donor antigens results in activation of the transcription of cytokine messenger RNAs (mRNA) followed by the subsequent cytokine protein release. These soluble mediators regulate the immune response against the transplanted graft. Human T-cells, although not so tightly restricted as described for mouse T-cells, can be divided into functionally different subsets on basis of their cytokine production pattern. Th0 cells produce cytokines such as IL-2 and IL-4, while Th1/Tc1 cells secrete IL-2, IFN-γ, TNF-ß and Th2/Tc2 cells produce IL-4, IL-5, IL-6, IL-10 (4,5). Allograft rejection is associated with an upregulation of several cytokines within the graft implying that Th0 cells or a mixture of both Th1/Tc1 and Th2/Tc2 cells have

infiltrated the allograft (6,7). The production of cytokines within the graft can be analyzed directly in biopsies on the transcriptional level and on the protein level using techniques such as RT-PCR and immunohistochemistry. It was shown that, in man, cardiac allograft rejection is associated with intragraft production of IL-2 and IFN-γ at both the transcriptional and protein level (8-10). Also the frequency of IL-2 producing helper T-cells with specificity for donor-antigens in peripheral blood of heart transplant recipients was associated with rejection (11). In contrast, for the Th2 cytokines IL-4 and IL-10 the data are less clear. Also at the transcriptional level, we and others found that both during acute cardiac allograft rejection and during immunological quiescence IL-4 and IL-10 mRNA expression can be found within the graft (8,9). This suggests that while these cytokines may be present during allograft rejection they actually may be involved in the down-regulation of the allogeneic immune response. Indeed, a role for IL-4 in the process of spontaneously resolving rejection and even in tolerance has already been reported after clinical liver and renal transplantation (12,13). Although it is thus clear that both IL-2 and IL-4 are important determinants of outcome of the immune process in human transplants, the kinetics of Th1 and Th2 cytokine production by graft infiltrating lymphocytes (GIL) and by peripheral blood mononuclear cells (PBMC) at the mRNA and at the protein level in the graft is unknown. We have shown that *in vivo* maturated, primed, T-cells with specificity for donor antigens home in the allograft, while naive, precursor T-cells are primarily found in the peripheral blood (14). The capacity of *in vivo* "primed" GIL and of "naive" PBMC to produce cytokines can be determined after *in vitro* challenge with donor antigens (15). In order to study the regulation of cytokine production by GIL and by PBMC it is essential to measure cytokine mRNA expression since cytokine production is predominantly controlled at the transcriptional level (16). An important advantage of our *in vitro* system is that mRNA expression by these cells can be compared directly with the cytokine protein production. Therefore, we have analyzed the kinetic pattern of IL-2 and IL-4 mRNA synthesis by *in vivo* "primed" GIL, grown from rejection biopsies, and by simultaneously obtained "naive" PBMC after *in vitro* stimulation with donor and third party antigens using semi-quantitative RT-PCR analysis. These findings are

compared directly with levels of these cytokines measured in cell culture supernatants by ELISA.

MATERIALS AND METHODS

Patients

Endomyocardial biopsies (n=3) and simultaneously taken PBMC (n=1) were obtained heart transplant recipients during their first acute clinical rejection episode (14-84 days posttransplant). Maintenance immunosuppressive therapy consisted of cyclosporin A and low-dose steroids. No additional therapy was given. Rejection was histologically diagnosed according to the criteria of the International Society for Heart and Lung Transplantation (3). For the diagnosis of clinical significant rejection, the co-existence of myocyte damage and mononuclear infiltrates was required (grade 3A). In that case, anti-rejection therapy was instituted.

GIL culture method

GIL cultures were grown from endomyocardial biopsies in 96 wells U bottom tissue culture plates (Costar, Cambridge, MA) as described before (17). GIL were expanded in culture medium (RPMI-1640 Dutch Modification Gibco, Paisley, Scotland) supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU penicillin and 100 µg/ml streptomycin) in the presence of 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF, Biotest AG, Dreieich, Germany) and 10^5 irradiated (30 Gy) autologous peripheral blood mononuclear cells. GIL cultures were grown at 37 °C in a humidified CO_2 incubator for two to three weeks. Half of the medium was refreshed every two to three days.

Phenotypic analysis of GIL cultures

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

Allogeneic stimulator cells

B lymphoblastoid cell lines (B-LCL) originated from infection of donor and third party spleen cells with EBV obtained from the marmoset cell line B95-8 (18).

Study design

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

Total RNA preparation

Total RNA was isolated from the stimulated GIL and their controls by a modification of the Chomczynski and Sacchi method as described before (9,19,20). Briefly, cells were homogenized in 500 µl 4 M guanidinium-isothiocyanate in the presence of 20 µg poly A (Boehringer Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamyl alcohol [25:24:1] and chloroform-isoamyl alcohol [24:1], respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000 x *g* at 4°C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice.

cDNA synthesis

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

PCR amplification and Southern blot analysis

Sequence specific primers were used for amplification of the human cytokine genes (table 1). PCR primers detecting transcripts for the human house keeping gene keratin, which is expressed by hematopoietic cell (21), were used as an internal control to monitor mRNA extraction and cDNA amplification (table 1). For qualitative analysis, 5 µl cDNA sample was added to 95 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a

DNA thermal cycler (Biomed-60, Germany) under the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) for 4 h at 37°C. Messenger RNA from this positive control was extracted as described above. Negative controls consisted of omission of reverse transcriptase from the cDNA synthesis reaction for each EMB followed by amplification in PCR with the IL-2, IL-4 and keratin primers, and the use of and diethylpyrocarbonate-treated H_2O as no-template reaction. After amplification, 1 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, Aylesbury, UK) by electroblotting and hybridized with $\gamma^{32}P$ labelled specific probes. Hybridization was detected by autoradiography.

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

Target	Sequence	Product size (bp)
IL-2 sense	5'ATG TAC AGG ATG CAA CTC CTG TCT T3'	458
IL-2 anti-sense	5'GTC AGT GTT GAG ATG ATG CTT TGA C3'	
IL-2 probe	5'TTC TTC TAG ACA CTG AAG ATG TTT CAG TTC3'	
IL-4 sense	5'ATG GGT CTC ACC TCC CAA CTG3'	462
IL-4 anti-sense	5'TCA GCT CGA ACA CTT TGA ATA TTT CTC TCT CAT3'	
IL-4 probe	5'GTC CTT CTC ATG GTG GCT GTA GAA CTG CCG3'	
Keratin sense	5'TGA AGA TCC GTG ACT GGT AC3'	218
Keratin anti-sense	5'ATG TCG GCT TCC ACA CTC AT3'	
Keratin probe	5'TCT CCT TCT GCA GAT TGA CAA TGC CCG TCT3'	

Semi-quantitative RT-PCR

For semi-quantitative analysis, cDNA samples were titrated (10-fold dilutions) and aliquots of each dilution were amplified using the conditions as described above. The amount of target cDNA present was expressed as end-point of the titration. Corrections were made for the integrity of the mRNA isolated and the efficiency of cDNA synthesis, which both may vary from isolation to isolation by standardization with keratin. It was expressed as arbitrary mRNA equivalents, defined as the highest

dilution showing positive signal. To estimate the relative initial amount of functional mRNA in the GIL cultures, aliquots of titrated cDNA samples were amplified using specific primers for keratin to correct for the above mentioned variables. The results were expressed as a ratio calculated from the titre of IL-2 or IL-4 gene PCR product over the titre of amplified keratin product. To exclude inter- and intra-assay variability, amplification of titrated cDNA of PHA-M activated spleen cells were included in each assay. These control cells expressed keratin, IL-2 and IL-4.

Enzyme linked Immunosorbent assay (ELISA)

The concentration of cytokines in the supernatants of the GIL cultures was measured with the following ELISA kits: IL-2 (Immunotech, Marseille, France) detection range 15-1000 pg/ml IL-2; IL-4 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) detection range 10-450 pg/ml IL-4.

Statistics

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

RESULTS

Phenotypic analysis of GIL cultures

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

mRNA expression of the housekeeping gene keratin

This transcript was chosen as a marker for the total number of cells and for the total quantity of mRNA extracted. Keratin mRNA expression is found to be in the range of mRNA expression of the cytokines tested, which is an advantage above the frequently used house keeping genes ß-actin and glyceraldehyde 3-phosphate dehydrogenase (20,22). Especially in muscle tissue glyceraldehyde 3-phosphate dehydrogenase levels are abundant and therefore not suitable for analysis in heart samples (23). All samples obtained from one culture were isolated at the same time.

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

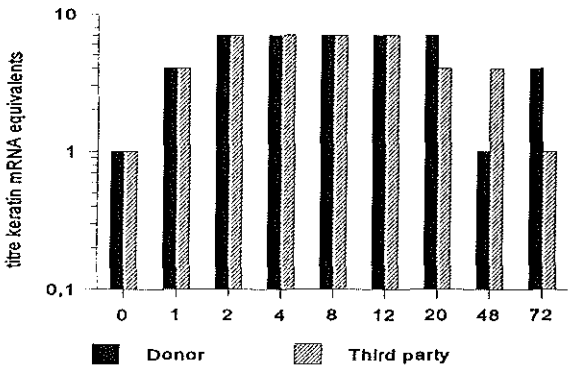


Figure 1. Time course of keratin mRNA expression in GIL cultures (n=3) after stimulation with either donor (black bars) or third party antigens (hatched bars). To estimate the relative initial amount of target cDNA, aliquots of ten-fold diluted cDNA samples were amplified using specific primers for keratin.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

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

In the present study, IL-2 and IL-4 mRNA expression was analyzed only in GIL cultures and irradiated stimulator B-LCL with keratin mRNA expression. Figure 2 depicts the time course of keratin, IL-2 and IL-4 mRNA expression in culture III after stimulation with donor and third party cells. Table 2 shows the kinetics of IL-2 and IL-4 mRNA expression of each individual GIL culture after stimulation with donor and third party antigens respectively. Unstimulated GIL cultures (0 h), and irradiated stimulator B-LCL alone neither expressed mRNA nor secreted protein of IL-2 and IL-4 at detectable levels (table 2 and 3). Upon stimulation by cells of donor or third

party origin an upregulation of cytokine mRNA production was observed. In general, IL-2 and IL-4 mRNA was apparent as early as 1 h after stimulation and reached maximal levels within 2 - 48 h after which it gradually decreased to prechallange base-line levels (20 -72 h). This pattern was seen after stimulation with both donor and third party antigens. However, the relative amount of IL-2 and IL-4 mRNA produced by GIL was higher after donor specific stimulation than after stimulation with third party cells (p=0.02). Moreover, the relative amount of donor specific IL-2 or IL-4 mRNA by these GIL cultures was clearly different. GIL cultures grown from endomyocardial biopsies taken during acute rejection synthesized significantly more donor specific IL-2 mRNA than IL-4 mRNA (p=0.002). In 2 out of 3 cultures, we measured a 100-fold IL-2/IL-4 mRNA ratio of the maximum mRNA levels.

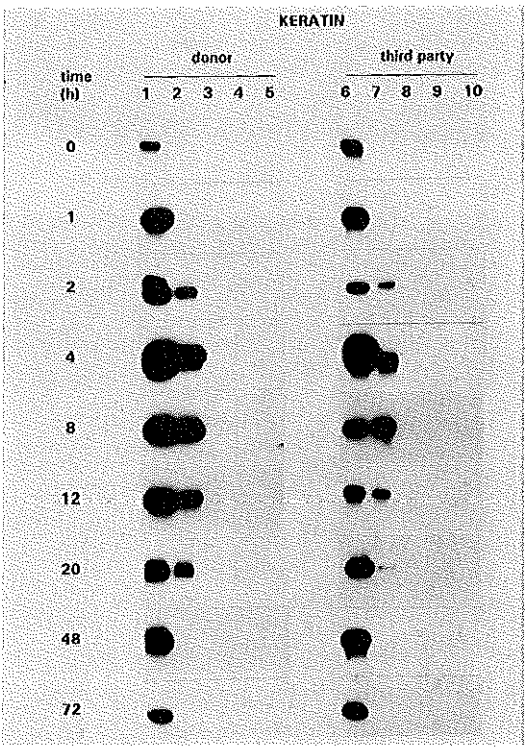
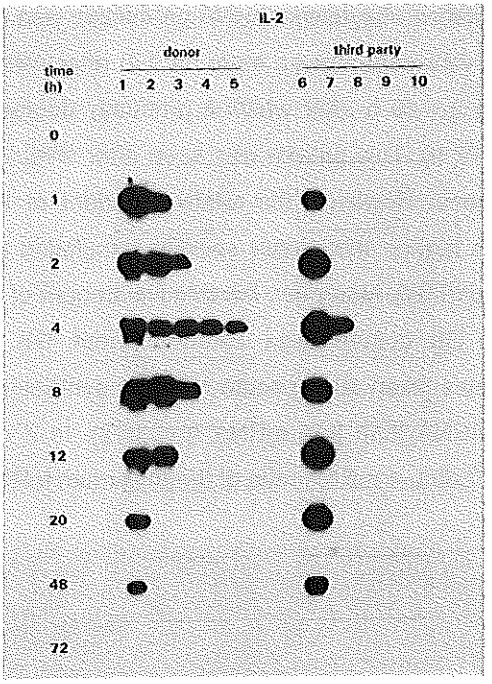


Figure 2. Semiquantitative RT-PCR analysis of keratin, IL-2 and IL-4 mRNA expression by Southern blotting and hybridization.


IL-2
donor
third party
time (h)
1 2 3 4 5
6 7 8 9 10
0
1
2
4
8
12
20
48
72

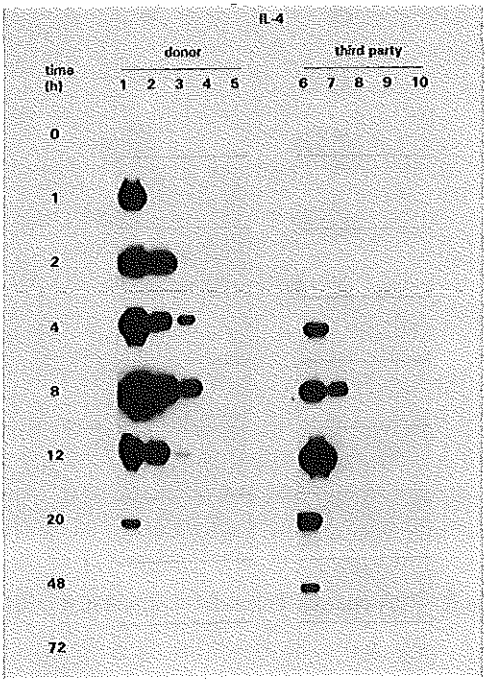

IL-4
donor
third party
time (h)
1 2 3 4 5
6 7 8 9 10
0
1
2
4
8
12
20
48
72

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

	donor stimulation						third party stimulation					
	I[1]		II		III		I		II		III	
time (h)	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4
0	-[2]	-	-	-	-	-	-	-	-	-	-	-
1	1[3]	0.1	1	-	10	1	10	0.1	-	-	1	-
2	1	1	10	1	10	1	10	1	-	-	0.1	-
4	1	1	10	10	1000	10	1	0.1	-	1	1	0.1
8	1	1	1	1	10	10	1	0.1	1	-	0.1	1
12	1	0.1	10	1	1	10	1	1	1	1	0.1	0.1
20	100	0.1	10	-	0.1	0.1	0.1	1	10	-	0.1	0.1
48	100	-	10	-	1	-	-	-	1	-	1	1
72	0.1	-	-	-	-	-	-	-	0.1	-	-	-

[1] GIL culture number

[2] not detectable

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

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

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

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

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

	donor stimulation						third party stimulation					
	I[1]		II		III		I		II		III	
tome (h)	IL-2[2]	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4
0	<15[3]	<10	<15	NA	<15	NA	<15	<10	<15	NA	<15	NA
1	<15[4]	<10	<15		<15		<15	<10	<15		<15	
2	<15	<10	<15		<15		<15	<10	<15		<15	
4	62	<10	<15		70		48	<10	<15		<15	
8	115	<10	28		117		35	<10	<15		<15	
12	161	<10	35		136		43	<10	<15		<15	
20	849	16	24		131		50	<10	<15		<15	
48	524	15	29		224		55	21	<15		<15	
72	164	<10	20		125		35	<10	<15		<15	

[1] GIL culture number

[2] pg/ml

[3] detection limit

[4] N.A., not available

The overall IL-2 protein was significantly higher after stimulation with donor than after third party stimulation (p=0.002, Wilcoxon signed rank test).

Relative IL-2 mRNA and protein levels by PBMC

In PBMC, messengers coding for IL-2 were detectable 2 h, and for IL-4 28 h after stimulation with donor antigens and 20 h and 1 h, respectively, after stimulation with third party antigens (table 4). Neither the relative IL-2 mRNA level nor the relative IL-4 mRNA level was significantly different between donor and third party stimulation, but only donor-specific stimulation resulted in measurable IL-2 protein levels at 48 h. Similarly to GIL, also in the PBMC, the relative maximum donor-specific IL-2 mRNA level was significantly higher compared to the relative donor-specific maximum IL-4 mRNA level (100-fold).

Table 4. IL-2 and IL-4 mRNA and protein production by PBMC[1] after stimulation with donor B-LCL and third party control B-LCL

	donor stimulation				third party stimulation			
	mRNA[2]		protein[3]		mRNA		protein	
	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4
time (h)								
0	-[4]	-	<15[5]	<10	-	-	<15	<10
1	-	-	<15	<10	-	1	<15	<10
2	1	-	<15	<10	-	1	<15	<10
4	1	-	<15	<10	-	-	<15	<10
20	1	-	<15	<10	100	-	<15	<10
28	10	-	<15	<10	100	1	<15	<10
48	100	10	82	<10	100	1	<15	<10

[1] PBMC of patient I

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

[3] pg/ml

[4] not detectable

[5] detection limit

DISCUSSION

After transplantation, it is thought that allogeneic primed T-cells are responsible for the process leading to graft destruction. After allogeneic stimulation these cells produce cytokines. However, the kinetics of donor-specific IL-2 and IL-4 mRNA and subsequent protein production by these "primed" and also by "naive" T lymphocytes is not known. Therefore, we have analyzed the kinetics of IL-2 and IL-4 mRNA and subsequent protein production by *in vivo* "primed" GIL, grown from rejection biopsies, and by "naive" PBMC after *in vitro* stimulation with donor and third party cells. In time, the induction of both mRNA and protein was quicker by GIL than by PBMC. Especially in GIL cultures, the relative level of IL-2 mRNA transcription and subsequent IL-2 protein production was significantly higher after stimulation with

donor antigens. This relative high level of IL-2 production by GIL cultures may reflect increased transcription in each cell or is the result of a higher frequency of donor specific IL-2 producing T-cells in the graft during rejection. This explanation is in line with the results of Bishop et al. who showed a high frequency of IL-2 producing T-cells in sponge matrix allografts (24). The results for IL-4 mRNA expression are in agreement with that of IL-2 mRNA. Also, at the mRNA level a donor-specific IL-4 signal was measured. However, during rejection, GIL cultures synthesized significantly more donor specific IL-2 mRNA than IL-4 mRNA. We could not detect these primed T-cells in PBMC on basis of significant early donor-specific mRNA transcription and subsequent protein production by the highly sensitive RT-PCR and ELISA, respectively. Probably the alloantigen primed T-cells are not or below the detection level present in the peripheral blood. These findings suggest that *in vivo*, "primed" T-cells with donor specificity mediated early events of the immune response within the transplanted organ.

Cells cultured from the transplanted graft are stimulated *in vivo* by donor antigen on antigen presenting cells and as a consequence they are differentiated into either Th1 or Th2 cells and can not be influenced by addition of cytokines as is the case for naive T-cells (25). Upon restimulation *in vitro* these cells maintain a similar pattern of cytokine production. Moreover, both IL-2 (Th1) and IL-4 (Th2) producing cells proliferate in response to the added IL-2 *in vitro* (26). Accordingly, it is likely that most of the *in vitro* cytokine production is released by GIL that differentiated *in vivo* into either Th1 (IL-2) or Th2 (IL-4) producing lymphocytes. Also after transplantation, the Th1/Th2 paradigm is frequently used to explain different immunological phenomena. Abundant production of Th1 cytokines is often accompanied with low secretion of Th2 cytokines and visa versa (6,7). Our results obtained in human lymphocytes at time of cardiac rejection are consistent with the hypothesis of the balance between IL-2 and IL-4 in allogeneic processes. Much of today knowledge about the kinetics of cytokine production by lymphocytes is based on studies of clonal cells and peripheral blood cells after stimulation with either mitogens or alloantigens (27-31). These studies show that IL-2 and IL-4 mRNA expression is rapidly but transiently induced after stimulation in T-cells. Also, in our bulk GIL

cultures IL-2 and IL-4 mRNA transcription was detectable as early as 1-2 h after stimulation reached maximum levels between 2 - 48 h and base-line levels were approached by 20 - 72 h. Subsequent IL-2 protein release was detectable from 4 h with cytokines accumulating at least 20 - 48 h. In contrast to the IL-2 mRNA expression by mitogen activated human T-cells as described by Shaw and colleagues (27) our mRNA profiles were definitely less spiking, which might be due to the composition of our bulk cultures. Phenotypic analysis showed that both $CD4^+$ and $CD8^+$ T-cells were present in the bulk GIL cultures. However, the time-course patterns of IL-2 and IL-4 production on the mRNA and protein level by $CD4^+$ and $CD8^+$ T-cells are only marginally different (28). In contrast, the kinetics of cytokine mRNA production by lymphocytes is dependent on their activation level (32). Accordingly, we assume that in the graft, during acute rejection, cytokines are produced by allospecific T-cells with different stages of activation. After the initial priming, alloreactive T-cells home in the graft but nevertheless, represent, less than 1% of the total infiltrate (14,33). Severe local inflammation, perhaps triggered by cytokine release might not only recruit T-cells with allospecificity to the graft but also T-cells with broader or irrelevant specificity. This mechanism might also explain why considerable levels of IL-2 and IL-4 mRNA expression were also found after stimulation with third party cells. In the present study, the time course patterns of mRNA expression of IL-2 and IL-4 were comparable between GIL stimulated with donor cells and those activated third party cells, suggesting that also infiltrating T-cells with more public specificities underwent some level of activation. Nevertheless, these third party cytokine mRNA levels, in contrast to donor specific cytokine mRNA levels, did not regularly result in measurable protein levels. A strong correlation has been shown between mRNA expression and protein production of various cytokines in GIL cultures isolated from rejected kidneys after activation with anti-CD3 (35). Anti-CD3 antibodies will activate all infiltrated T-cells including the donor-specific subpopulation. However, *in vivo* T-cell activation requires the interaction of antigen presenting cells, peptide and a specific T-cell receptor. Therefore, the results after *in vitro* stimulation with alloantigens is a better reflection of events within the allograft during acute rejection. The discrepancy we found between mRNA and protein

production after third party but not donor-specific stimulation could of course be a detection phenomenon. Another explanation is that donor-specific signals are involved at the post-transcriptional level. GIL have T-cell receptors with low affinity for third party antigens and activation of these low affinity T-cell receptors might not lead to all the necessary signals required for translation of mRNA (35).

In conclusion, the kinetics of cytokine mRNA and protein production was significantly different between "naive" PBMC and "primed" GIL. In addition, analysis showed that each individual GIL culture had its own characteristic mRNA and protein profile presumably due to differences in activation level of the alloreactive T-cells. Only these *in vivo* maturated primed T-cells are capable of secreting IL-2 (Th1) and IL-4 (Th2) immediately upon antigen stimulation.

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Chapter 4

CYTOKINE mRNA EXPRESSION IN ENDOMYOCARDIAL BIOPSIES DURING ACUTE REJECTION FROM HUMAN HEART TRANSPLANTS

This chapter is based on the article published in:

Clin Exp Immunol 1994;97:293-298

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ABSTRACT

The immune response to an allograft is regulated by cytokines produced by cells infiltrating the allograft. However, the immunopathogenesis of allograft rejection is not completely understood. To investigate the role of cytokines after clinical heart transplantation, we analyzed the expression of cytokine genes in sequentially taken endomyocardial biopsies (EMB) by using the reverse transcriptase-polymerase chain reaction (RT-PCR). We analysed 44 EMB from 11 recipients: 21 EMB before or during rejection, and 23 EMB without histological evidence of acute rejection. A strong correlation was found between IL-2 gene expression and histologically proved rejection (16/21 versus 1/23 without rejection; $p<0.001$; X^2-test). Also, expression of IL-4 and IL-6 genes was more often found in EMB during rejection than in EMB without signs of rejection (IL-4, 62% versus 35%; and IL-6, 81% versus 39%, respectively). No relation with rejection or with immunological quiescence was observed for the presence of IL-10 gene transcripts. IL-10, but also IL-6 mRNA were detectable in donor heart tissue before transplantation (9/10). In contrast, IL-2 and IL-4 gene transcripts were absent in these samples. These differences could not be explained by the presence or absence of T-cells, since the gene for the constant region of the ß-chain (Cß) of the T-cell receptor (TCR) not only was expressed in post-transplant EMB but also in pre-transplant donor heart tissue.

Our results provide strong evidence that the immunoregulatory cytokines IL-2, IL-4 and IL-6 are important local regulators in the graft during acute rejection. The role of IL-10 in the immunologic response to the transplanted organ needs further investigation.

INTRODUCTION

The interaction of immunocompetent cells with their targets causes changes in the steady-state expression of different cytokine genes. Cytokines are soluble mediators of communication and it has become clear that these regulators can be secreted both by graft infiltrating cells and by cells from graft tissue. Evidence is increasing that different immunologic situations after organ transplantation may be associated

with specific patterns of cytokine gene expression, although discrepancies in the detection between mRNA for certain cytokines and the specific cytokine protein have been reported (1). In case of rejection, cytokine gene expression is observed even before tissue damage as reflected by histology or functional impairment of the graft is detected. Intragraft studies using Northern blotting and *in situ* hybridization following experimental and clinical transplantation showed that allogeneic reactions may be associated not only with expression of IL-2, IL-4 and interferon-γ (IFN-γ) (2,3) but also with gene expression of proinflammatory cytokines such as IL-1ß, tumour necrosis factor-α and IL-6 (4,5,6). In contrast, IL-10 and probably IL-4 might play a major role in suppressing rejection and inflammatory responses (7,8). Although useful, both Northern blotting and *in situ* hybridization require relatively large amounts of mRNA and are technically difficult. In contrast, the reverse transcriptase-polymerase chain reaction (RT-PCR) provides a sensitive and reproducible method for the analysis of expression of several cytokine genes in a small number of cells. Using this technique, local transcription of various interleukin mRNAs was found during clinically relevant rejection episodes. In human kidney transplants an association was found with IL-2 expression while in liver transplants IL-5 seemed to be more important (9,10,11). After clinical lung transplantation, cytokine gene analysis on broncho alveolair lavage material showed that the IL-4 gene expression is more prominent at the time of a cellular rejection period (12). The situation after heart transplantation is less obvious. Studies in experimental animals, cynomolgus monkeys and mice demonstrated that IL-2, IFN-γ, IL-4 and IL-6 gene transcription was induced within the transplanted heart during the processes of rejection (13,14). In contrast, in human cardiac allografts a correlation was found between histological rejection and expression of IL-6 and transforming growth factor-beta (TGF-ß) genes (15,16) which could be the result of non-specific inflammation rather than a T-cell mediated specific allogeneic reaction.

In order to assess cytokine transcription patterns that might be related with cell mediated acute rejection or immunological quiescence after clinical heart transplantation, we determined the presence of IL-2, IL-4, IL-6 and IL-10 gene transcripts in donor heart tissue and in endomyocardial biopsies (EMB) post-

transplantation by RT-PCR. By the same methodology, expression of the constant region of the ß chain (Cß) gene of the T-cell receptor (TCR) was performed to analyse the expression of cytokine genes in relation with the presence of a T-cell infiltrate. The results were correlated with histological findings on simultaneously taken EMB.

MATERIAL AND METHODS

Patients

We analysed 44 EMB from 11 heart transplant recipients who showed histological signs of rejection. All EMB were taken during the first year after transplantation before, during and after rejection. Donor heart tissue taken before transplantation served as control (n = 10). Patients received cyclosporin A and low-dose steroids only as maintenance immunosuppressive therapy. Rejection was histologically diagnosed in EMB by the criteria of the International Society for Heart and Lung Transplantation (17). In brief: grade 0, no evidence for rejection; grade 1A, focal (perivascular or interstitial) infiltrate without necrosis; grade 1B, diffuse but sparse infiltrate without necrosis; grade 2, one focus only with aggressive infiltration and/or focal myocyte damage; grade 3A, diffuse inflammatory process with necrosis; grade 3B, diffuse inflammatory process with necrosis; grade 4, diffuse aggressive polymorphous ± infiltrate, ± edema, ± hemorrhage, ± vasculitis, with necrosis. For this study, EMB were grouped into those without myocyte damage (grade 0 and 1, n = 23), and those with myocyte damage (grade 2 and 3, n = 21).

RNA extraction and transcription

Donor heart tissue and post-transplant EMB for RT-PCR were snap-frozen in liquid nitrogen and stored at - 80°C until analysis. Total RNA was extracted from snapfrozen EMB samples by a modification of the guanidinium method as described by Chomczynski and Sacchi (18). EMB tissue was homogenized in 500 µl 4 M guanidine thiocyanate in the presence of 10^4 mouse 3T3 cells (ATCC, Rockville, MD). These mouse cells act as a carrier to improve the yield of total RNA. The solution was extracted with phenol, phenol-chloroform-isomethylalcohol [25:24:1] and chloroform-isomethylalcohol [24:1]. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000xg at 4°C and washed with 500 µl cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from 25 µl of the isolated RNA with 0.25 µg hexanucleotides (0.5 µg/µl; Promega Corporation, Madison, WI) and transcribed with 2.5 µl Moloney murine leukemia virus (MMLV) reverse

transcriptase (200 U/µl; Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture contained 10 µl 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 µl dNTP (10 mM) 0.5 µl of RNAsin (400 U/µl; Promega) and 5 µl DTT (0.1M).

PCR amplification and Southern blot analysis

Sequence specific primers (Clontech Laboratories, Palo Alto, CA) were used for amplification of the human cytokine genes. These primers were located next to splice sites, to be able to discriminate for RNA only. Five µl cDNA sample was added to 45 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler (Biomed, thermocycler 60) under the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min. As positive controls for the cytokines we used cDNA isolated from HuT78 cells (ATCC, a human T-cell line derived from a patient with Sezary syndrome), and Epstein-Barr virus (EBV) immortalized B cells. The presence of T-cells was determined by amplification of the gene coding for the ß chain constant region of the TCR as described by Hawes (19). PCR primers detecting transcripts for the human keratin gene (sense primer 5'TGAAGATCCGTGACTGGTAC3' and anti-sense primer 5'ATGTCGGCTTCCACACTCAT3') were used as an internal control to confirm successful RNA extraction and cDNA amplification (20). After amplification, 8 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, Aylesbury, UK) by electro blotting and hybridized with $\gamma^{32}P$ labelled probes which are located across the splice-site (cytokines, Clontech Labs; and keratin, 5'TGTCCTTCTGCAGATTGACAATGCCCGTCT3'). Hybridization was detected by autoradiography.

RESULTS

We analysed 10 samples of pre-transplant donor graft tissue and 44 post-transplant EMB, obtained from 11 recipients for alterations in expression of IL-2, IL-4, IL-6 and IL-10 genes in relation to histological diagnosis. Transcripts from the human keratin gene were amplified in all tested samples. mRNA extracted from the mouse 3T3 carrier cell line did not show expression of genes coding for human cytokines, nor for Cß of the TCR or keratin.

Pre-transplant donor cardiac tissue

Genes coding for IL-6 and IL-10 were expressed in the majority of the pre-transplant samples, whereas IL-4 mRNA was never detectable and IL-2 mRNA was present in one sample only (table 1). Also Cß mRNA of the TCR was successfully amplified in 8 of the 10 pre-transplant samples (table 1), reflecting the presence of the T-cells in donor cardiac tissue.

Table 1. Cytokine and TCR-Cß chain gene expression in pre-transplant donor heart tissue. Southern blot analysis of polymerase chain reaction (PCR)-amplified DNA

Donor	IL-2	IL-4	IL-6	IL-10	TCR-Cß chain
I	-[a]	-	+	-	+
II	-	-	+	+	+
III	-	-	+	+	-
IV	-	-	+	+	+
V	-	-	+	+	+
VI	+	-	+	+	+
VII	-	-	+	+	+
VIII	-	-	+	+	+
IX	-	-	-	+	-
X	-	-	+	+	+
+/total	1/10	0/10	9/10	9/10	8/10

[a] -, no signal; +, positive expression

Cytokine gene expression versus histologic evidence of rejection

Typical results of the kinetics of cytokine gene expression in pre-transplant and post-transplant EMB for one patient before, during and after acute cellular rejection are shown in figure 1. Results of sequential EMB from 11 patients are presented in tables 2 and 3 for EMB without and with histological evidence of rejection, respectively. The presence of IL-2 transcripts was characteristic for EMB taken at the time of rejection as 16/21 EMB (76%) showed transcription of the IL-2 gene, while only 1/23 (4%) EMB in which no myocyte damage was seen, expressed the IL-2 gene ($p<0.001$; X^2-test, tables 2 and 3). IL-2 mRNA transcripts were already

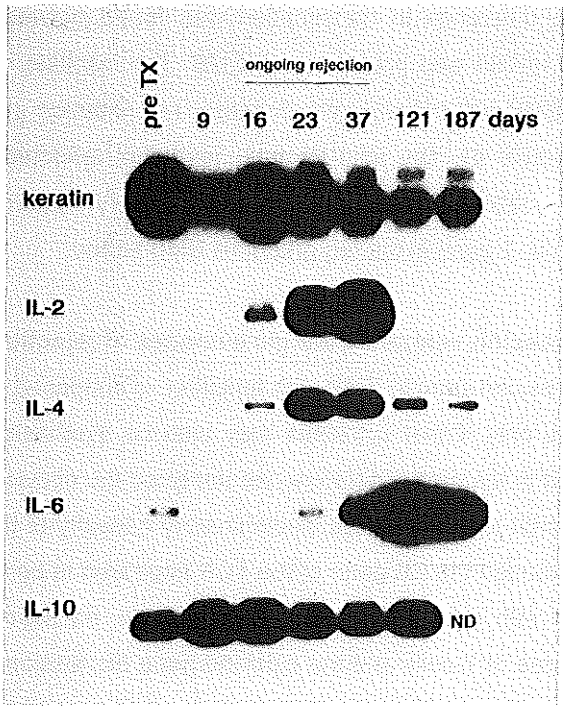

Figure 1. Southern blot analysis of RT-PCR-amplified keratin, IL-2, IL-4, IL-6 and IL-10 transcripts from mRNA obtained from one patient (Me) of pre-transplant and post-transplant EMB, taken before, during and after rejection. Pre TX, pre-transplant EMB; day 9 post-transplant (pTX), no rejection; day 16 pTX, one focus only with aggressive infiltration and/or focal myocyte damage; day 23 pTX, one focus only with aggressive infiltration and/or focal myocyte damage; day 37 pTX, diffuse inflammatory process with necrosis; day 121 pTX, focal infiltrate without necrosis; day 187 pTX, no rejection.

detectable in EMB with histological rejection grade 2, preceding grade 3 EMB (table 3). Prior to or during an acute rejection episode, IL-2 mRNA positive EMB frequently showed coexpression both of IL-4 and IL-6 mRNA (10/16). Nevertheless, the presence of IL-4 gene transcripts did not discriminate between EMB with and those without histological signs of rejection (13/21, 62% vs 8/23, 35%; $p>0.05$: tables 2 and 3). Neither was the presence of IL-6 mRNA solely indicative for episodes of rejection, since in these cases 17/21 EMB (82%) expressed the IL-6 gene compared to 9/23 EMB (39%) taken at the time of immunological quiescence (table 2 and 3).

For IL-10 gene no difference in transcription was found between the two EMB groups since IL-10 mRNA was present in all EMB. The cytokine mRNA profiles of the subgroups of EMB without myocyte damage, i.e. rejection grades 0 and 1A, were comparable (table 4). The mild immune response reflected in EMB by the presence of cellular infiltration but without myocardial injury, apparently did not lead to expression of the IL-2 gene in the ten grade 1A EMB tested.

TCR-Cß gene expression versus histologic evidence of rejection

The IL-2 gene expression seen in EMB with rejection, is supposed to be transcribed by activated T-cells only. Other cytokines may also be synthesized by cell types present concomitant in the graft. Therefore, we analysed the occurrence of T-cells in the EMB in an attempt to enhance the discriminatory capacity of the other cytokine RT-PCR results. Although the Cß gene of the TCR appeared to be more prominently expressed in EMB with rejection than in those without, no significant correlation between the presence of T-cells and the cytokine gene expression pattern could be made. TCR-Cß gene transcription was detectable in all 44 post-transplantation EMB, those with and those without rejection and even in pre-transplant donor heart tissue (fig 2).

DISCUSSION

After transplantation, cytokines are transcribed and produced both by cells from graft tissue and by lymphocytes infiltrating the allograft. Cytokines mediate and control the allogeneic response, and therefore monitoring of cytokine gene patterns might be helpful in understanding immunological processes in the transplanted heart. Therefore, we determined cytokine and TCR-Cβ gene expression in pre-transplant donor heart tissue and in post-transplant EMB.

We have shown that the intragraft cytokine gene pattern of post-transplant donor cardiac tissue differs from that of normal tissue. We found that pre-transplant heart tissue contained IL-6 and IL-10 transcripts.

Table 2. Cytokine gene expression in endomyocardial biopsies (EMB) without histological evidence of rejection[a]. Southern blot analysis of PCR-amplified DNA

EMB[b]	Days post-transplant	IL-2	IL-4	IL-6	IL-10
Bi1	16	-[c]	-	+	+
Bi4	155	-	-	+	ND
Bo1	28	-	-	-	+
Bo3	230	-	-	-	+
De1	8	-	+	-	+
De4	100	-	+	-	+
Ge2	44	-	+	+	ND
Ge3	51	-	-	+	ND
Ko1	31	-	-	-	ND
Ko3	105	-	-	-	ND
Me1	9	-	-	-	+
Me5	121	-	+	+	+
Me6	187	-	+	+	ND
Mi1	22	-	-	-	ND
Pe3	346	-	-	-	+
Po1	22	-	+	+	+
Po3	58	-	-	-	+
Po5	126	+	+	+	+
Se1	19	-	-	-	+
Se4	80	-	-	-	+
Se5	111	-	+	+	+
Se6	262	-	-	-	ND
Wa4	135	-	-	-	ND
+/total		1/23	8/23	9/23	14/14

[a] No rejection: EMB grading 0 and 1 according to Billingham (17)

[b] EMB description: composed of patient identification and EMB number analyzed

[c] ND = not done; -, no signal; +, positive cytokine expression

Table 3. Cytokine gene expression in endomyocardial biopsies (EMB) with histological evidence of rejection[a]. Southern blot analysis of PCR-amplified DNA

EMB[b]	days post-transplant	IL-2	IL-4	IL-6	IL-10
Bi2	60	-[c]	+	-	+
Bi3	67	+	-	-	+
Bo2	125	+	+	+	ND
De2	29	+	+	+	+
De3[d]	86	+	+	+	+
Ge1	16	+	-	+	ND
Ge4	72	+	-	+	ND
Ge5	101	+	-	+	ND
Ko2	99	-	-	+	ND
Me2[d]	16	+	+	-	+
Me3[d]	23	+	+	+	+
Me4	37	+	+	+	+
Pe1	59	+	+	+	+
Pe2	114	-	-	-	+
Po2	35	+	+	+	+
Po4	96	-	+	+	+
Se2[d]	26	+	-	+	+
Se3	33	+	+	+	+
Wa1	16	+	+	+	ND
Wa2	23	-	-	+	ND
Wa3	108	+	+	+	ND
+/total		16/21	13/21	17/21	13/13

[a] Rejection: EMB grading ≥ 2 according to Billingham (17)

[b] EMB description: composed of patient identification and EMB number analyzed

[c] ND = not done; -, no signal; +, positive cytokine expression

[d] Rejection grade 2 biopsies

In contrast, we hardly ever detected IL-2 and IL-4 gene expression in these samples. Therefore, the presence of these latter transcripts appeared to be characteristic for the immune response after transplantation. We found that local IL-2 mRNA transcription was correlated with cellular rejection leading to myocyte damage, after clinical heart transplantation. Moreover, analysis showed that IL-2 mRNA transcripts were already detectable in EMB histologically defined as rejection grade 2, in the period preceding rejection defined as rejection grade 3. Our results after clinical heart transplantation are in agreement with RT-PCR data after experimental heart transplantation (8,13,14,21). The discrepancy between these data and those published by Zhao et al (15,16) may be the result of differences in the amount of cardiac tissue from which the RNA was isolated or in sensitivity of the RNA extraction. In contrast to the studies by Zhao et al, we added carrier cells at the beginning of the RNA extraction which is necessary for optimal isolation from small amounts of tissue (22,23). Also their identification of a PCR product was solely based on the generation of a PCR product of predicted size while the identity must be verified by a γ ^{32}P labeled internal probe which is significantly more specific and sensitive. At this point one has to keep in mind that detection of mRNA using sensitive RT-PCR techniques may not give a correct impression of relevant production of the biologically active cytosine proteins. One could for instance hypothesize a (post)-translational blockade due to the clinical use of immunosuppressants, although such an activity of cyclosporin or prednisone has not been described. It would nevertheless be important for our understanding of the cytokine network in transplantation medicine if we could correlate mRNA expression with protein production. This, however, implies the introduction of an *in vitro* cell culturing phase with all it's adherent confounding variables. The prominent place for IL-2 in the clinical rejection process as suggested by our RT-PCR results has also been found by Ruan et al (24) in their immunohistochemical study. As in our present report they showed that IL-2 was absent or only occasionally expressed in EMB without signs of myocyte damage. In our study, some of the tested EMB taken during rejection did not express the IL-2 gene.

Table 4. Cytokine gene expression in the subgroups of endomyocardial biopsies (EMB) without myocyte damage

Gene product	rejection grade 0 n[#] (%[1]) (n = 13)	rejection grade 1A n (%) (n = 10)
IL-2	1 (7)	0 (0)
IL-4	4 (31)	4 (40)
IL-6	5 (38)	4 (40)

[#] number of positive EMB

[1] Positive EMB as percentage of total EMB tested

Apparently sampling timing is essential and might be explained by the transient expression of IL-2 (25). Besides, focal expression may cause discordance between EMB tested for cytokine gene expression and concurrently taken EMB obtained for histological evaluation. Activated lymphocytes not only release IL-2 but also other cytokines such as IL-4 (26). Like IL-2, IL-4 was also only expressed in post-transplant EMB, but its presence had low predictive value for acute allograft rejection. Still the finding that IL-2 and IL-4 genes are concurrently expressed during acute rejection indicates that not only IL-2 but also IL-4 plays an important role in the regulation of the allogeneic response *in vivo*. This is in agreement with the observations by Dallman et al (14) and by Morgan et al (21) who reported that in non-immunosuppressed murine cardiac allografts genes for IL-2 and IL-4 are induced during rejection. The difference between the more clear-cut role for IL-4 in these experimental studies and our less uniform findings might be the result of immunosuppressive therapy in the clinic. Also recently published data of human lung transplant recipients demonstrated that induction of IL-4 gene transcription is far from uniformly in allograft rejection (12). In contrast to the mRNA for IL-2 and IL-4, the presence of the IL-6 and IL-10 mRNA was not confined to post-transplant EMB. The proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 are not produced by activated T-cells only (27,28) but are synthesized by a variety of cells,

including monocytes (29,30) and endothelial cells (31,32) which are normally present in cardiac tissue. The proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 are not produced by activated T-cells only (27,28) but are synthesized by a variety of cells, including monocytes (29,30) and endothelial cells (31,32) which are normally present in cardiac tissue.

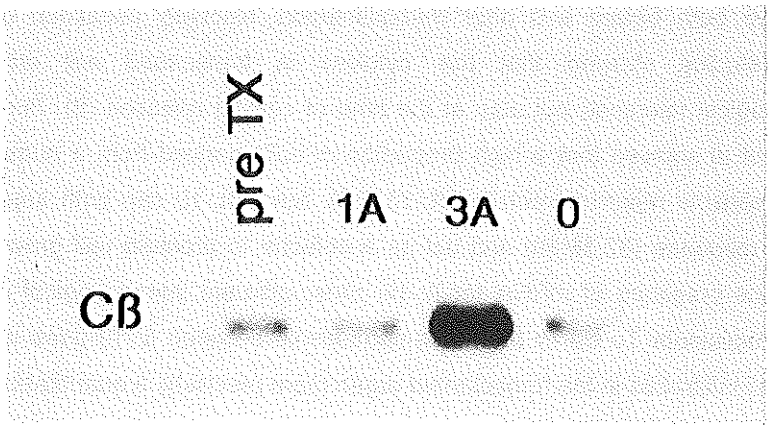

Figure 2. Southern blot analysis of RT-PCR-amplified ß-chain transcripts of the constant region (Cβ) of the T-cell receptor from mRNA obtained from one patient (Ko) of pre-transplant allograft tissue and post-transplant endomyocal biopsies (EMB). Histology: pre TX = donor heart tissue before transplantation; day 31 post-transplant (pTX), 1A: focal (perivascular or interstitial) infiltrate without necrosis; day 99 pTX, 3A: diffuse inflammatory process with necrosis; day 105 pTX, 0: no rejection.

The inflammation that accompanies surgery would suffice to induce release of IL-6 (33) and possibly other cytokines such as IL-10. In the current study, measurement of IL-6 mRNA directly in post-transplant EMB was associated with, but again had no predictive value for allograft rejection. IL-6 transcripts were present in 81% of the EMB with clinically relevant rejection (myocyte damage) and in only 40% of those with irrelevant rejection (infiltrate only). It is likely that IL-6 is involved in the inflammation process occurring during acute rejection since the presence of IL-6 transcripts in post-transplant EMB is associated with a massive infiltration of T-cells (fig 2) and monocytes (34) in the allograft which suggests that least some of the IL-6

gene expression is derived from cells invading the allograft. The positive correlation of IL-6 expression to acute renal graft rejection is supported by several studies (4,35,36). This relationship, however, was not found after clinical liver transplantation where intragraft IL-6 mRNA transcription was not associated with allograft rejection (10). Interestingly, in the present study, the IL-10 cytokine gene was persistently expressed both before and after transplantation. Recently published data from mouse studies showed that this cytokine is transcribed not only during immunological tolerance but also at the time of cellular rejection (8). Furthermore, Merville et al showed that a high proportion of cells derived from a human kidney with irreversible rejection secreted IL-10 (35). Since IL-10 can both suppress the immune response (37,38,39) and act as a cofactor to promote cell growth (40), a continuous presence of mRNA for IL-10 under different immunological conditions can be explained. To clarify the exact relationship between IL-10 gene expression and T-cell infiltration in the transplanted organ more precisely, a quantitative or semi-quantitative analysis is required.

In conclusion, rejection and immunological quiescence were correlated with distinct patterns of cytokine gene expression. We found a clear correlation of IL-2 and to a lesser extent of IL-4 and IL-6 gene expression with myocyte damage. These cytokine profiles might be of prognostic significance for rejection processes after clinical heart transplantation. However, the role of IL-10 in processes leading to rejection or immunological tolerance remains to be determined.

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Chapter 5

BLOCKADE OF THE IL-2/IL-2RECEPTOR SIGNALLING PATHWAY WITH A MONOCLONAL ANTI-INTERLEUKIN-2 RECEPTOR ANTI-BODY (BT563) DOES NOT PREVENT THE DEVELOPMENT OF ACUTE HEART ALLOGRAFT REJECTION IN HUMANS

This chapter is based on articles published in:

Transplantation 1998;65:405-410

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Transplant Int (submitted)

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ABSTRACT

Anti-Interleukin-2Receptor (IL-2R) antibodies have been used as rejection prophylaxis after organ transplantation. Despite this induction treatment acute rejections may occur. We wondered whether these rejections developed via the IL-2/IL-2R pathway. In a prospective trial using BT563, a murine IgG_1 anti IL-2Receptor antibody, for rejection prophylaxis after heart transplantation, 20 patients were treated in combination with cyclosporine from the day of transplantation (group A). As a control group served 31 patients also treated with BT563, but in these patients cyclosporine treatment was initiated on day 3 (group B).

Three patients from group A and two patients from group B died in the first post-operative month (of causes not related to acute rejection) and were left out from the analysis of rejection incidence. Freedom from acute rejection at 1 week after transplantation in group A (14/17; 82%) was lower than in group B (16/29; 55%), although the difference did not reach statistical significance. There was no difference in either the number of acute rejections at 12 weeks or the required rejection treatments between groups A and B. Infectious complications were evenly distributed in both groups. Immunohistochemistry showed that during acute rejection, in the presence of circulating BT563, IL-2R bearing cells were present in only 1/5 (20%) rejection biopsies, whereas these cells were often present (75%; 7/8) in rejections occurring in the absence of BT563. BT563 presence was associated with a similar difference in the mRNA expression of IL-2 (40% versus 75%). In contrast, no effect of BT563 was seen on intragraft mRNA transcription of the macrophage derived T-cell growth factor IL-15. Quantitative analysis in rejection biopsies showed comparable IL-15 mRNA levels during and after BT563 treatment.

Despite adequate blockade of the IL-2/IL-2R signalling pathway patients may develop acute rejection, reflecting the redundancy of the cytokine network. The ever-present IL-15 may well be a candidate for overtaking the role of IL-2.

INTRODUCTION

OKT3, an anti-pan-T-cell monoclonal antibody, is effective as anti-rejection therapy and

possibly as rejection prophylaxis in heart and kidney transplantation (1,2). However, the administration of OKT3 is accompanied by a cytokine release syndrome and a profound non-specific immunosuppression that may result in an increased incidence of infectious complications and development of malignancies (3-5). More specific immunosuppression may be obtained by use of monoclonal antibodies against the interleukin-2 Receptor (IL-2R MoAbs). Anti-IL-2R MoAbs administration is not complicated by a cytokine release syndrome. Clinical trials with several examples of this class of antibodies have been performed in the prevention of acute rejection after kidney, combined kidney and pancreas, heart and liver transplantation (6-13).

Our earlier controlled trial comparing safety and efficacy of BT563, a murine IgG_1 anti-IL-2R MoAb, with OKT3 as prophylaxis against acute rejection after heart transplantation showed no difference between the two groups with respect to freedom from rejection at 3 months or infectious complications (11). During BT563 treatment, however, a high incidence of early acute rejections was found. In contrast, a significant reduction of early acute rejection episodes was found in a trial with BT563 to prevent acute rejection in kidney transplant recipients (9). An important difference between our heart and kidney transplant trial is that in the renal allograft recipients cyclosporine treatment started on day 0, whereas in the heart transplant patients, cyclosporine was initiated on day 3 to prevent nephrotoxicity in the first post-operative days. In animal studies, a synergistic effect of the combination of cyclosporine and anti-IL-2R MoAbs has been demonstrated (14,15). The results of rejection-prophylaxis with BT563 in the heart recipients might have been better if cyclosporine had been initiated on the day of transplantation, making optimal use of the synergistic effect of combining BT563 and cyclosporine. The results of our renal study support this hypothesis (9).

In this article, we present data of a study investigating the effect of combining cyclosporine and BT563 from the first day after heart transplantation on rejection incidence, renal function and adverse side effects. Apart from clinical data, immunological monitoring of the IL-2/IL-2R pathway in peripheral blood by flow cytometry, and within the graft by immunohistochemistry and by reverse transcriptase-polymerase chain reaction (RT-PCR) is presented.

PATIENTS AND METHODS

Patients

Between February 1996 and November 1996, 20 consecutive recipients of a cardiac allograft (group A) were prophylactically treated with BT563 after returning from the operation theatre. BT563 (Biotest Pharma GmBH, Dreieich, Germany) was given in a dosage of 11 mg i.v. from day 0 to day 12 after transplantation. In addition to BT563, patients were treated with prednisone 50 mg daily from day 0, gradually decreasing to 15 mg at 4 weeks after transplantation. Cyclosporine was started 6 hours after vascular anastomosis in a dosage of 2 mg/kg/day i.v. After 2 days oral cyclosporine administration was started, aiming at whole blood trough levels of 250 - 350 ng/ml (EMIT, Behring Diagnostics, Cupertino, CA). Endomyocardial biopsies (EMBs) were performed weekly during the first 6 weeks, biweekly during the next month, and monthly for the next 4 months after transplantation. Biopsies were repeated more frequently after a rejection episode.

As a control group (group B) we used the BT563-treated patients (n=31) of the earlier randomized trial, following an identical treatment and surveillance protocol, but receiving cyclosporine on day 3 after transplantation (11). These patients were transplanted between November 1991 and February 1994. The duration of BT563 administration for patients in group A was 12 days, and for group B, 7 days.

Peripheral blood monitoring

Before, during, and after BT563 treatment peripheral blood samples were collected at day 1, 3, 5, 7, 14, 21, 28, 35 and 42 in EDTA containing tubes and monitored for the presence of T-cell subsets by two color flowcytometry using MoAb directly conjugated to fluorescein (FITC) or phycoerythrin (PE). Before incubation with MoAb, 100 μl whole blood was washed three times with 2 ml Hanks' Balanced Salt Solution (Gibco, Paisley, Scotland) supplemented with 1% Bovine Serum Albumin (BSA) (Sigma, St.Louis, MO) and 0.1% sodium azide (Merckx, Darmstadt, Germany) to remove BT563 and human-anti-mouse antibodies that might interfere with the staining procedures. Subsequently, 20 μl of the following dual MoAb combinations were added to 100 μl cell suspensions: CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2a}-PE as isotype control; CD3-FITC/CD19-PE; and the combination WT31-FITC (anti-T-cell receptor [TCR] α/β) /CD25-PE (MoAb 2A3) all obtained from Becton & Dickinson (San Jose, CA). After 30 min of incubation at room temperature, red blood cells were lysed with FACS lysing solution (Becton & Dickinson) during 10 min. After washing, cells were analysed on a FACScan (B&D) flow-cytometer using SimulSet software (B&D) for data analysis. To establish an analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 reagent was used. One thousand to 2000 gated lymphocyte events were acquired from each tube. The CD25 MoAb 2A3 is also directed against the IL-2R α-chain, but is not competitive with BT563 (16). In all patients, we also analysed for the presence of CD122 [IL-2R ß-chain (p75)] on the T-lymphocytes by the MoAb combination TCR α/ß-FITC (WT31) and CD122 (anti IL-2R p75)-PE (B&D). A PE-conjugated monoclonal rat-anti-mouse antibody (RAM, B&D) was used to determine whether CD25-

positive cells in peripheral blood were coated with BT563 during treatment ("bound BT563").

Plasma BT563 levels

BT563 trough levels were measured daily during treatment. Plasma levels were determined with an ELISA sandwich method using goat anti-mouse-IgG (Southern Biological Associates, Birmingham, AL) and alkaline-phosphatase-(AP)-conjugated goat anti-mouse-IgG (H+L) antibodies (Jackson Immuno Research, West Grove, PA). Samples were diluted 1:450 and 1:2000 with Tris buffered saline/Tween 20/Bovine Serum Albumine (TBS/Tw/BSA). The concentration of BT563 was estimated by interpolation of obtained values on a standard curve of parental grade BT563 diluted in this solution.

Rejection: monitoring and treatment

A pathologist, unaware of the immunosuppressive protocol, examined EMB-specimens stained with hematoxylin-eosin using light microscopy. Biopsies were graded according to the guidelines of the International Society for Heart and Lung Transplantation (17). Biopsies graded 3A or higher were regarded to represent rejection episodes necessitating anti-rejection treatment. In the majority of cases rejection was treated with 1 gram methylprednisolone i.v. on three consecutive days. Frequently recurring or refractory rejection episodes were treated with rabbit-ATG (Imtix, Amstelveen, The Netherlands). Recurrent episodes of steroid-unresponsive rejection after treatment with r-ATG were treated with a course of OKT3.

Immunohistochemistry

From all patients in group A, immunohistochemistry was performed on all EMBs taken weekly until the first acute rejection episode or until week 6 for non-rejectors (n=48). From 11 patients, a peroperative time=0 EMB was obtained. Snap-frozen EMB specimens were cut in 5 μm sections, air dried, and fixed in 4% formalin. The slides were subsequently incubated with the appropriately diluted MoAb [CD3 = RIV9 (RIVM, Bilthoven, Netherlands); CD4 = anti-Leu3 (B&D); CD8 = anti-Leu2 (B&D); CD25 = 2A3(B&D); CD68 = mouse anti-human macrophage clone PK1 (DAKO, Denmark)]. The slides were rinsed in phosphate-buffered saline (PBS) and incubated with biotinylated antimouse-immunoglobulins (1:50 in PBS; Biogenex, San Ramon, CA), rinsed, and labeled with AP-labeled streptavidin (Biogenex, 1:50 in PBS). The enzym was finally detected with new fuchsin as a substrate as described by the manufacturer. Slides were counterstained with Mayer's haematoxylin, examined and the number of positive staining cells was counted. The results are expressed per half field at 100x magnification (obj. 10x). A total field at this magnification measures 2.54 mm^2. After staining the biopsies were scored on a scale from 0 to 4. Score 0: no positive cells; score 1: 1-10 positive cells; score 2: 11-30 positive cells; score 3: 31-100 positive cells; score 4: > 100 positive cells. "Bound" BT563 was measured using the above mentioned procedure, omitting the first step.

mRNA isolation and cDNA reaction

For RT-PCR analysis, specimens were snap-frozen in liquid nitrogen and stored at -80°C. mRNA extraction and transcription was performed as described previously (18). Briefly, total RNA was extracted from snap-frozen samples by a modification of the guanidinium method, as described by Chomczynski and Sacchi (19). Specimens were homogenized in 500 μl 4M guanidinium-isothiocyanate in the presence of 20 μg/ml poly A (Boehringer, Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1]. Total RNA was precipitated with 600 μl 2-propanol and 35 μl 3M sodium acetate (pH 5.2) at -20°C for 18 hr. Precipitates were pelleted at 10.000xg at 4°C during 20 min and washed once with 500 μl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 μl diethylpyrocarbonate (DEPC) treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from the isolated RNA with 0.5 μg hexanucleotides (Promega Corporation, Madison, WI) and transcribed with 1000 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 100 μl. The reaction mixture consisted of 20 μl 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 5 μl dNTP (10 mM), 400 U of RNAsin (Promega) and 10 μl (0.1M) DTT.

Polymerase Chain Reaction (PCR)

Sequence-specific primers were used for amplification of the human IL-2, IL-2R (α-chain), IL-4, IL-15 and TCR-Cß genes. PCR primers detecting transcripts for the human house-keeping gene ß-actin (Clontech Laboratories, Palo Alto, CA) were used as an internal control to monitor mRNA extraction and cDNA amplification. For qualitative analysis, 5 μl cDNA sample was added to 95 μl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence-specific primers. Each reaction mixture was overlaid with 75 μl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler (model 480, Perkin-Elmer, Norwalk, CT) using the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) for 4 h at 37°C. mRNA from this positive control was extracted as described above. Negative controls consisted of omission of template from the cDNA synthesis reaction for each biopsy followed by amplification in PCR with the IL-2, IL-2R (α-chain), IL-4, IL-15, TCR-Cß, and ß-actin primers, and the use of DEPC-treated-H_2O as no-template reaction. After amplification, 16 μl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, UK) by electroblotting, and hybridized with $\gamma^{32}P$ labelled specific probes which are located across the splice-site.

Hybridization was detected by autoradiography and indicated the presence of IL-2, IL-2R (α-chain), IL-4, IL-15, TCR-Cß and ß-actin mRNA expression in the original biopsy.

Competitive template RT-PCR

To determine the relative amount of functional IL-2R (α-chain), IL-15, and TCR-Cß mRNA in EMB, a competitive RT-PCR assay was used and comparison was made against the house-keeping ß-actin gene. This gene is assumed to be expressed at a constant level in EMB. To obtain a standard curve for IL-2R (α-chain), IL-15, TCR-Cß and ß-actin, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive amplification with specific primers. The internal control was designed to generate a PCR product of a different size to allow differentiation between the amplified target and internal standard. Dilutions of the competitor template, ranging from 0.1 fg to 5 pg in 5 μl, were coamplified with constant amounts of sample cDNA. After PCR, under conditions identical to those described above, the amplification products were analyzed by gel electrophoresis, and the amount of products by the internal control and targets are determined for each individual reaction. The relative ethidium bromide intensity on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, NY). The logarithm of the ratio target/internal control is plotted as a function of the logarithm of the internal amount of the standard. At ratio 1, the starting concentration of IL-2R (α-chain), IL-15, TCR-Cß and ß-actin cDNA before PCR is assumed to be equal to the known starting concentration the competing internal control. The relative concentration of intragraft IL-2R (α-chain), IL-15, and TCR-Cß gene transcripts were divided by the relative concentration of ß-actin. This represents the amount of IL-2R (α-chain), IL-15, and TCR-Cß mRNA transcripts corrected for the amount of mRNA used for reverse transcription and the efficacy of each reaction.

Statistics

For the determination of levels of statistical significance, two-sided probability values according to Fisher's exact test or, if appropriate, the Mann Whitney U test were calculated.

RESULTS

Clinical data

The baseline characteristics of the patients that entered this study are shown in table 1. The BT563-treated patients from this study (group A) were similar to the patients in the control group (group B) in respect to all of these characteristics.

The majority of recipients were male (82%), > 40 years of age (85%), and suffered from ischemic heart disease (65%). Most donors were < 40 years of age (83%). Donors and

recipients were not matched for cytomegalovirus (CMV) serology. CMV-positive donor / CMV-negative recipient combinations were equally distributed among the two groups.

Table 1. Baseline characteristics of heart transplant recipients

	Group A (n=20)	Group B (n=31)
Age (median;range)	55 (36-64)	53 (14-65)
Weight kg (median;range)	72 (58-91)	69 (34-114)
Gender (M/F)	17/3	25/6
Etiology heart disease:		
ischemic	12	20
cardiomyopathy	7	7
other	1	4
Donor age yrs (median;range)	33 (13-43)	27 (14-45)
Cold Ischemia time (min.)	163 (119-240)	155 (107-260)

Peripheral blood monitoring

Within hours after the first dose of BT563, the IL-2R-bearing cells (CD25) in peripheral blood were covered by the antibody (figure 1). Peripheral blood lymphocytes remained negative for CD25 for the duration of BT563 adminstration and for a period of 3 to 21 days (median, 7 days) thereafter. During this period CD122 (ß-chain) remained present, showing continued presence of IL-2R positive cells, but with a coated α-chain. BT563 trough levels reached a plateau phase after day 5, with mean trough levels of 6144 (± 445) ng/ml. The half life of BT563 in blood, calculated from the descending part of the curves, using a one-compartment model, was 39 ± 3 hr.

Clinical complications

As a result of starting cyclosporine 6 hr after transplantation, we observed cyclosporine nephrotoxicity in 7/19 (37%) of patients. In all cases this was reversible, but temporary interruption (in most patients 12-36 hr) or reduction in cyclosporine administration was

necessary. No patient required renal replacement therapy for cyclosporine nephrotoxicity. Mortality in group A was 3/20 in the first 3 months. One patient died on postoperative day 1 as a result of severe hemorrhage from a ruptured suture. One patient suddenly died in the postoperative week 4 after having had a fever for more than a week, for which no clear cause could be found and for which broad-spectrum antibiotic treatment had been initiated. A third patient died 26 days post-transplantation, after having developed renal and hepatic failure in a situation of primary donor-organ failure from the first day after transplantation. In group B, 2/31 patients died, 1 as a result of hemorrhagic stroke on day 4, the other due to sepsis after several thoracotomies for tamponade on day 17. These 5 patients were excluded from the analysis of rejection-incidence. Infectious complications were evenly distributed between the two groups. Urinary tract infections (7/29 versus 5/17), herpes hominis/zoster infections (7/29 versus 5/17) and CMV disease (3/29 versus 2/17) were most frequent.

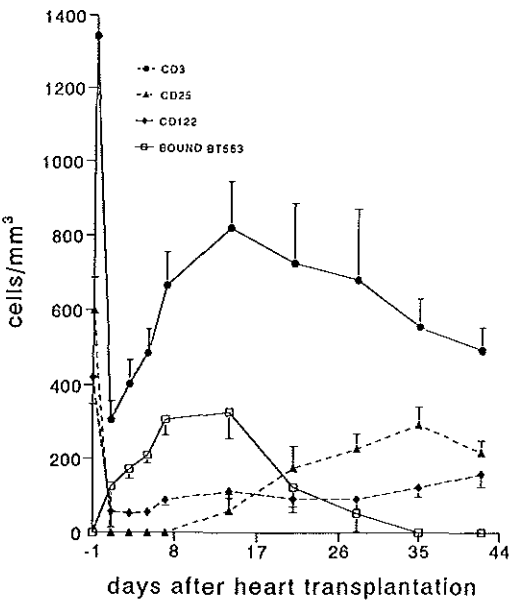

Figure 1. Peripheral blood monitoring during the first 6 weeks after heart transplantation. The first dose of BT563, given at day 0, results in a complete coverage of all CD25+ cells. The cells remain coated for the duration of BT563 treatment. The CD122 signal (IL-2R ß-chain) is proof of the fact that BT563 does coat the IL-2R α-chain (CD25) and not the ß-chain. After BT563 levels drop to 0 uncoated α-chains reappear. The strong decrease in the number of CD3+ cells reflects the effect of steroid treatment

Rejection

Figure 2 shows the numbers of patients free from rejection at several intervals after transplantation. The number of patients with two or more acute rejection episodes in group A and B were 8/17 (47%) and 17/29 (59%), respectively. Anti-rejection treatment given in group A (n=17) included 23 courses of steroids and 7 courses of anti T-cel antibodies (all rATG). In group B (n=29) 30 steroid-courses and 19 anti-T cel antibody courses (15 rATG, 4 OKT3) were used.

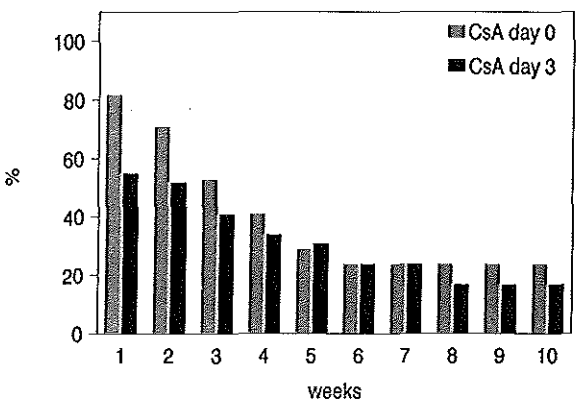

Figure 2. Freedom from acute rejection after heart transplantation. All patients received prophylactic treatment with an anti-IL-2Receptor antibody (BT563). Group A was treated with cyclosporin from day 0, in group B cyclosporine treatment was started on day 3.

Immunohistochemistry & RT-PCR

From 15/17 patients of group A, serial EMB were analysed immunohistochemically (n=59 EMBs). In 13/59 EMBs, acute rejection was present; in 36/59 EMBs represented non-rejection (ISHLT grade 0, 1A, 1B, or 2). In the rejection biopsies, CD25+ cells were present in 7/13 (54%). However, CD25+ cells could also be found in 13/36 (36%) of non-rejection biopsies. Among the rejection biopsies, the presence of CD25+ cells was clearly influenced by the presence of BT563: during presence of circulating BT563 cells bearing CD25 were found in only 1/5 (20%) rejection-EMBs, whereas CD25+ cells were present in 6/8 (75%) of rejection-EMBs after BT563 treatment. Using the quantitative immunohistochemistry score, significant differences in presence of numbers of CD3 and CD8 positive cells were found in rejection biopsies taken during or after BT563 treatment (figure 3).

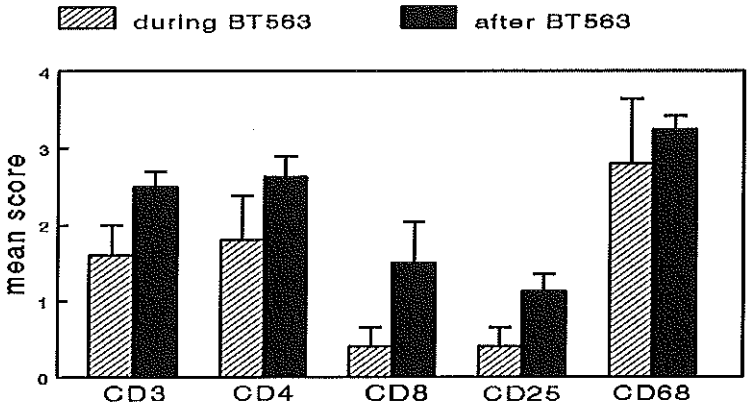

Figure 3. Quantitative immunohistochemistry of biopsies with an acute rejection during or after BT563 treatment. Significant differences between mean scores of biopsies during versus after BT563 treament were found for CD3 (p=0.04; Unpaired Student's t-test) and CD8 (p=0.004).

RT-PCR analysis of the same EMBs showed the presence of intragraft mRNA expression for IL-2 and IL-4 during rejection in 8/13 (62%) and 9/13 (69%), respectively. In non-rejecting EMBs, IL-2 and IL-4 mRNA expression was found significantly less frequently: 8/35 (23%; p=0.001) for both. Circulating BT563 antibody influenced the expression of IL-2 mRNA in rejection biopsies: during BT563 IL-2 mRNA expression was found in 2/5 (40%) vs 6/8 (75%) after BT563 treatment. For IL-4, these percentages were 2/5 (40%) and 7/8 (88%). Messenger coding for the IL-15 gene was constitutively expressed in all EMBs, regardless of rejection-status. For ß-actin, mRNA levels were comparable in EMBs during and after BT563 induction therapy (Mann-Whitney U test). With competitive template RT-PCR techniques, the relative amount of functional IL-2R and TCR-Cß mRNA compared against the house-keeping gene ß-actin were estimated. As shown in figure 4, rejection biopsies taken during BT563 treatment contained a factor 3 to 4 less IL-2R mRNA than rejection biopsies that occurred after BT563 treatment.

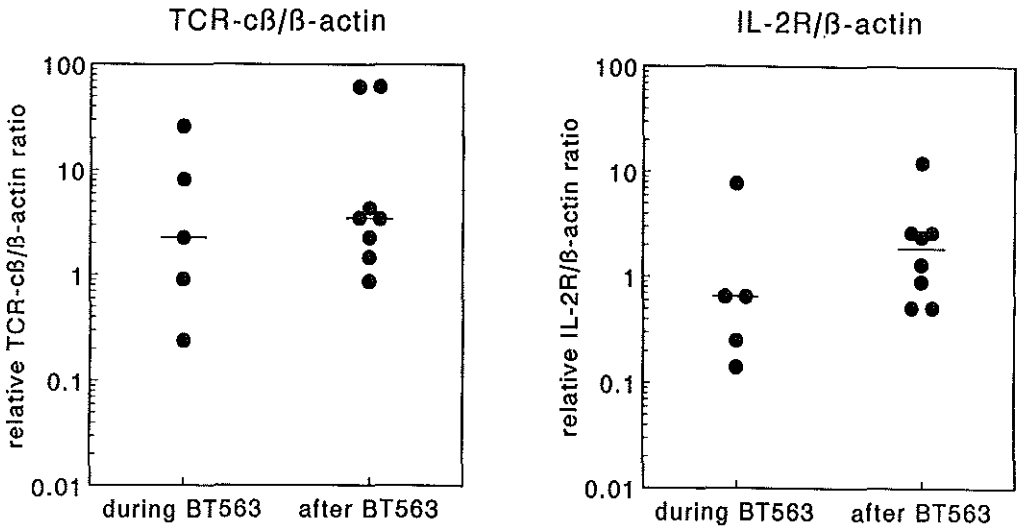

Figure 4. Quantitative RT-PCR results showing a higher amount of IL-2R mRNA in acute rejection biopsies taken during BT563 treatment compared to acute rejection biopsies taken after BT563 treatment.

IL-15 mRNA expression was found in all rejection EMBs, irrespective of BT563 treatment. Therefore, we performed quantitative RT-PCR analysis in order to detect an *in vivo* effect of BT563 on intragraft IL-15 mRNA levels. However, using the competitive template RT-PCR, we found comparable IL-15 mRNA levels in rejection EMB during and after BT563 treatment (figure 5).

DISCUSSION

In previous studies with the anti-IL-2R MoAb BT563, we showed that within 1 hr after the administration of BT563, no IL-2R α-chain positive cells can be detected in peripheral blood (20). Despite this clear effect of BT563 on peripheral blood lymphocytes, EMBs taken at the end of the prophylactic treatment period showed acute rejection in 13/29 (45%) patients of a previously performed study of prophylactic

treatment with this anti-IL-2R MoAb (group B) (11). In view of a study using BT563 induction treatment after kidney transplantation, in which a significant decrease in the incidence of early acute rejections was found, and in view of data from animal experiments where a synergistic effect was found for the combination of cyclosporine and anti-IL-2R MoAbs, the patients from group A in this study received BT563 induction treatment combined with cyclosporine from day 0 after heart transplantation (9, 14,15). As is shown in figure 2, the incidence of acute rejection in group A was (non-significantly) lower compared to that in group B. Again, acute rejections were found in the absence of IL-2R positive cells in peripheral blood. Immunohistochemistry of the rejection biopsies showed CD25+ cells in 7/13 (54%) EMBs. In the EMBs with rejection occurring during BT563 induction treatment, CD25+ cells were only present in 1/5 (20%). Using RT-PCR on EMB material, we found positive IL-2 mRNA expression in 8/13 (62%) of rejecting patients. In acute rejection EMBs during BT563 treatment, however, IL-2 mRNA could be shown in only 2/5 (40%). In contrast, immunohistochemistry of the EMBs of the patients in group B demonstrated the presence of CD25+ cells in 8/10 (80%) of these biopsies during acute rejection (21).

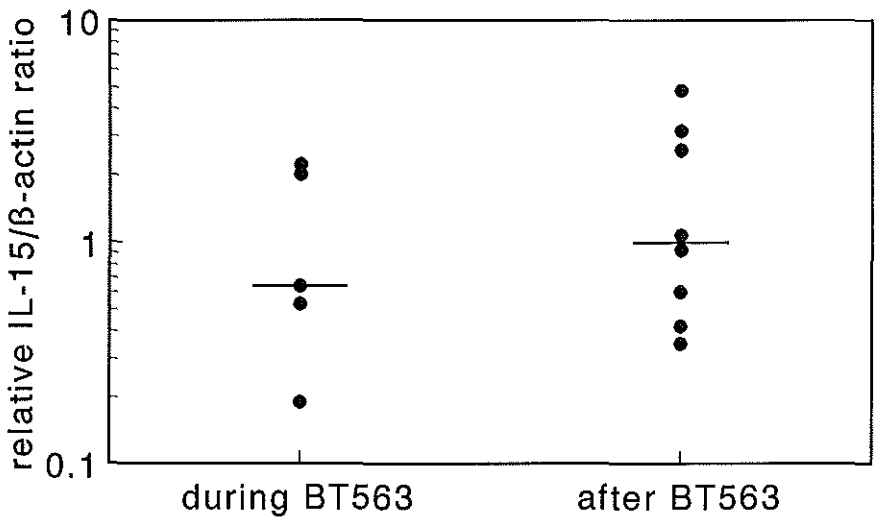

Figure 5. Relative mRNA levels are depicted as ratio of ß-actin. Quantitative RT-PCR analysis showed comparable intragraft IL-15 mRNA levels in rejection EMB during and after BT563 treatment.

Clearly, the administration of cyclosporine from day 1 after transplantation has resulted in a stronger blockade of the IL-2 pathway. However, this also means that apparently blockade of the IL-2/IL-2R complex alone is not sufficient to prevent patients from rejecting allografts. This parallels the observation that IL-2 knock-out mice are capable of allograft rejection as well (22,23).Other cytokines apparantly take over the activation of the rejection cascade, demonstrating again the difficulties we experienced as a result of the redundancy of the cytokine network. Others have suggested IL-15 to be a potentially important cytokine in this respect (24). IL-15 and IL-2 have overlapping biological functions. IL-15 mediates its function through the ß- and γ-chain of the IL-2R and its own IL-15α chain (25). In contrast to IL-2 and IL-4, IL-15 is produced by macrophages and other non-T-cells. During cardiac rejection, these macophages (CD68+ cells) are abundantly present within the graft, suggesting that IL-15 can participate in this process (figure 3). The MoAb we used was murine and non-humanized. Whether our results would have been better had a humanized antibody been used is uncertain. Up to now, published data on humanized anti IL-2R MoAbs are scarce (26,27). Data presented at the 16th annual meeting of the American Society of Transplant Physicians in Chicago in May 1997 showed excellent efficacy of these agents with respect to the prevention of acute rejection after kidney transplantation. We found effective blockade of the IL-2/IL-2R pathway with our MoAb. It is unlikely that blockade of this pathway by a humanized antibody would improve the results. However, there are indications that humanized antibodies do not only act through receptor site blockade, but also by destruction of activated T-cells by antibody dependent cytotoxicity (27). If that mechanism of action contributes strongly to the effects of humanized monoclonals that would explain their effectiveness in a redundant cytokine network.
In conclusion, despite combining cyclosporine and an anti-IL-2R MoAb (BT563) from day 1 after heart transplantation, we found an acute rejection rate of 39% in weeks 1 and 2. Most of these rejections occurred in the absence of activated lymphocytes expressing IL-2 and/or IL-2R. Apparently, other cytokines such as IL-15 have taken over the activation of the rejection cascade.

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Chapter 6

THE INTRAGRAFT CYTOKINE mRNA PATTERN REFLECTS THE EFFICACY OF STEROID ANTI-REJECTION THERAPY

This chapter is based on articles published in:

J Heart Lung Transplant 1996;15:1184-1193

Carla C. Baan, Hubert G.M. Niesters, Aggie H.M.M. Balk, Bas Mochtar, Pieter E. Zondervan, and Willem Weimar

Transplant Proc 1996;28(6):3239-3240

Carla C. Baan, Hubert G.M. Niesters, Aggie H.M.M.Balk, Bas Mochtar, Pieter E. Zondervan, Teun van Gelder, and Willem Weimar

ABSTRACT

We studied the effect of anti-rejection therapy on intragraft cytokine mRNA expression. Therapy consisted of 3 doses of 1 g of intravenous methylprednisolone (MP). We determined its effect on intragraft mRNA expression of immunoregulatory (IL-2, IL-4) and inflammatory cytokines (IL-1ß, IL-6, TNF-α), and the high affinity IL-2Receptor (IL-2R, p55 chain) in endomyocardial biopsy (EMB) specimens from cardiac allograft recipients.

By reverse-transcriptase-polymerase chain reaction (RT-PCR) methods, we detected mRNA transcription for IL-2 in 56% of the pre-treatment EMB (n=16), for IL-4 in 31%, and for IL-6 in 56% of the specimens, and IL-2R, IL-1ß, IL-6, TNF-α were constitutively expressed. Individual cytokine mRNA profiles were not helpful in differentiating between rejections that proved to be either MP resistant (n=9) or MP sensitive (n=7). After successful anti-rejection therapy, the overall intragraft mRNA expression was downregulated. None of the post-treatment EMB taken from six patients with MP sensitive rejections expressed the IL-2 gene, in contrast to 88% of the EMB obtained from eight patients with MP resistant rejections (p=0.005). Moreover, intragraft IL-4 and IL-6 mRNA transcripts were hardly detectable (both 17%) in MP reversible rejections, but in ongoing rejections IL-4 mRNA expression was found in 62% (p=0.14), and for IL-6 in 88% of the EMB (p=0.03). Semiquantitative analysis showed that the intragraft IL-2R, IL-1ß and TNF-α mRNA levels were lower in post-treatment EMB from MP reversible rejections than in EMB from MP irreversible rejections (p=0.03).

Our data suggest that the efficacy of anti-rejection therapy with MP is reflected in intragraft cytokine mRNA profiles.

INTRODUCTION

Glucocorticosteroids (GC), like methylprednisolone (MP) are powerful immunosuppressive and anti-inflammatory agents routinely used to prevent and treat allograft rejection (1,2). However, many patients fail to respond to high dose i.v. systemic MP anti-rejection therapy (3). Nevertheless, more than 30 years after the introduction of

GC as anti-rejection therapy, its *in vivo* immunosuppressive action on events within the transplanted organ is poorly defined (4). Analysis of these events might provide more insight in the *in vivo* mode of action of steroids and may contribute to a better understanding of mechanisms controlling steroid resistant rejection episodes.

In vitro studies have shown that GC inhibit the expression of cytokine mRNA from both lymphocytes (IL-2, IFN-γ) and antigen presenting cells (IL-1, IL-6, TNF-α, [5-10]). Also mRNA coding for the high affinity IL-2Receptor (IL-2R, p55 chain) is inhibited (11). GC bind to intracytoplasmatic receptors that translocate GC to the nucleus, where the steroids affect the glucocorticosteroid response element and/or activating protein-1 binding site (2,5,12). This blocks the transcription of cytokine genes, and the production of cytokines is suppressed. Analysis of intragraft mRNA expression by reverse-transcriptase-polymerase chain reaction (RT-PCR) methods showed that, after clinical heart transplantation, the inflammatory cytokines (IL-1ß, IL-6, TNF-α), the immunoregulatory cytokines (IL-2, IL-4), and the high affinity IL-2R are upregulated in the transplanted organ during rejection (13-16).

No clinical data are available on the *in vivo* inhibitory effects of anti-rejection therapy with MP at the transcriptional level of these cytokines within the transplanted organ. A different cytokine mRNA pattern may be expressed, or the baseline level of cytokine mRNA transcription may be different between steroid-sensitive and steroid-resistant rejections. In patients with asthma or rheumatoid arthritis, steroid resistance has been associated with simultaneous production of IL-2 and IL-4 (17,18). Alternatively, the differential effects of MP on the inhibition of the cytokine mRNA transcription *in vivo* may underlie the immunosuppressive efficacy in rejection. We analyzed local cytokine mRNA expression by RT-PCR in endomyocardial biopsy specimens (EMB) before and one week after start of the first MP anti-rejection therapy. We measured intragraft mRNA expression of IL-2, IL-2R (p55 chain), IL-4, IL-1ß, IL-6, and TNF-α, and the expression of mRNA coding for the constant region of the ß chain of the T-cell receptor (TCR-Cß) in relation to the expression of the cellular house keeping keratin gene.

PATIENTS AND METHODS

Heart transplant recipients

Patients underwent transplantation between 1992-1994 and received early prophylactic immunosuppression with BT563, a murine anti-interleukin-2 receptor monoclonal antibody (CD25, Biotest Pharma, Dreieich, Germany) or with OKT3 (Ortho Pharmaceutical Corp, Raritan, NJ). Maintenance immunosuppressive therapy consisted of cyclosporin A (whole blood trough levels were kept between 350-500 ng/ml in the first post-operative months) and low-dose steroids. No additional therapy was given. Rejection was histologically diagnosed in EMB according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT, [19]). Rejection therapy was instituted in case of moderate rejection (i.e., diffuse inflammatory process and myocyte damage, ISHLT grade 3A). Anti-rejection therapy consisted of 1 g MP administrated intravenously on 3 consecutive days. Steroid resistant rejection was defined, after one course of MP by persistent inflammatory infiltrates combined with myocyt damage of a similar or greater degree than that by which the diagnosis of cellular rejection was initially made. For the present study, we analysed EMB from 16 patients before (pre-treatment) and one week after start of MP therapy (post-treatment control EMB). We only studied biopsy specimens obtained from the first acute rejection episode. We analysed one specimen before and one specimen after MP anti-rejection therapy from each patient. Post-treatment control EMB demonstrated seven rejections that were steroid sensitive and 9 that were steroid resistant. One patient died within 3 months pTX from bacterial infection and was excluded from the analysis of the one year rejection incidence.

RNA preparation and cDNA synthesis

For RT-PCR analysis, EMB were snap-frozen in liquid nitrogen and stored at -80°C. Messenger RNA extraction and transcription was performed as described previously (15,16). Briefly, total RNA was extracted from snap-frozen samples by a modification of the guanidinium method as described by Chomczynski and Sacchi (18). EMB were homogenized in 500 µl 4 M guanidinium-isothiocyanate in the presence of 20 µg poly A (Boehringer Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1], respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000xg at 4°C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from 25 µl of the isolated RNA with 0.25 µg hexanucleotides (Promega Corporation, Madison, WI) and transcribed with 500 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture contained of 10 µl 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 µl dNTP (10 mM), 200 U of RNAsin (Promega) and 5 µl (0.1M) DTT.

PCR amplification and Southern blot analysis

Sequence specific primers (Clontech Laboratories, Palo Alto, CA) were used for amplification of the human cytokine genes. The presence of T-cells was determined by amplification of the gene coding for the ß chain constant region of the TCR (15). PCR primers detecting transcripts for the human house keeping gene keratin were used as an internal control to monitor mRNA extraction and cDNA amplification (15). All primers were located next to splice sites to be able to discriminate for mRNA only. For qualitative analysis, 5 µl cDNA sample was added to 95 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler (Biomed-60, Germany) under the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) for 4 h at 37°C. Messenger RNA from this positive control was extracted as described above. Negative controls consisted of omission of reverse transcriptase from the cDNA synthesis reaction for each EMB followed by amplification in PCR with the complete range of cytokine primers, and the use of and diethylpyrocarbonate treated-H_2O as no-template reaction. After amplification, 16 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, Aylesbury, UK) by electroblotting and hybridized with $\gamma^{32}P$ labelled specific probes which are located across the splice-site (15). Hybridization was detected by autoradiography. EMB were screened for IL-2, IL-2R (p55 chain), IL-4, IL-1ß, IL-6, TNF-α and TCR-Cß mRNA expression.

Semiquantitative RT-PCR

For semiquantitave analysis, cDNA samples were titrated (10-fold dilutions) and aliquots of each dilution were amplified using the conditions as described previously. The amount of target cDNA present was expressed as end point of the titration. Corrections were made for the integrity of the mRNA isolated and the efficiency of cDNA synthesis, which both may vary for each isolation. It was expressed as arbitrary mRNA equivalents, defined as the highest dilution showing positive signal. To estimate the relative initial amount of mRNA in EMB, aliquots of titrated cDNA samples were amplified using specific primers for keratin to correct for the described variables. To determine the relative amount of TCR-Cß, IL-2R, IL-1ß, and TNF-α mRNA in EMB, the level of target mRNA was corrected for the expression of the keratin gene, which was assumed to be expressed in a constant level in the EMB.

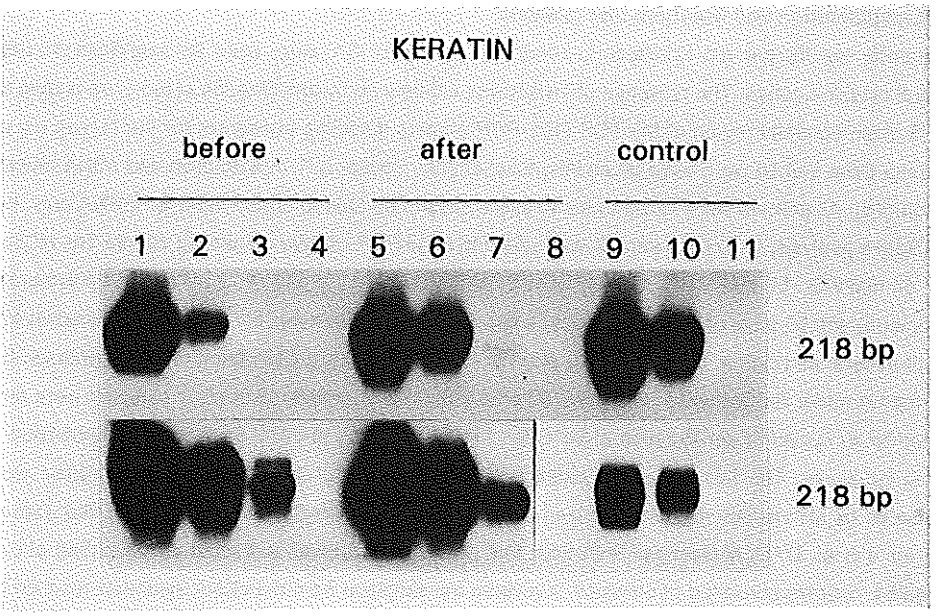

Figure 1. Comparison of keratin mRNA expression in EMB specimens from two patients before and after MP anti-rejection therapy and positive control (PHA-M activated spleen cells). Semiquantitative analysis of keratin mRNA expression in EMB obtained from a patient with a steroid-resistant first rejection episode (top) and from a patient with a steroid-sensitive first rejection episode (bottom). Lane 1 through 4 keratin mRNA expression before MP-treatment: lane 1, undiluted DNA; lane 2, 10-fold diluted DNA; lane 3, 100-fold diluted DNA; lane 4, 1000-fold diluted DNA. Lane 5 through 8, keratin mRNA expression after MP treatment: lane 5, undiluted DNA; lane 6, 10-fold diluted DNA; lane 7, 100-fold diluted DNA; lane 8, 1000-fold diluted DNA. Lane 9 through 11 positive control, lane 9, undiluted DNA; lane 10, 10-fold diluted DNA; lane 11, 100-fold diluted DNA. Before and after MP treatment the titres of keratin mRNA in different EMB derived from one patient were comparable, showing the integrity of the mRNA of these specimens.

Controls

To exclude interassay and intraassay variability, amplification of titrated DNA of PHA-M activated spleen cells were included in each assay. These control cells expressed all cytokines tested. Experiments with a variation in the titre of more than one 10-fold dilution step of keratin or cytokine mRNA levels in the positive control were excluded. Relative yields of keratin mRNA were comparable between EMB taken before (n=16) and after (n=14) MP treatment, indicating an equivalent of intact mRNA isolated in each EMB. Before and after MP treatment we measured a median keratin level of 10 input

mRNA equivalents (ranged between 10 to 100 input mRNA equivalents; p=0.72, Mann Whitney U test). This indicated that MP had no effect on mRNA expression of the house-keeping gene keratin. Moreover, before and after MP treatment the difference in the titre of keratin mRNA between individual EMB derived from one patient varied not more then one dilution step, showing the integrity of the mRNA in these specimens (figure 1). The keratin mRNA levels were converted to equal arbitrary keratin mRNA equivalents in to verify that equal amounts of mRNA were compared. Relative amounts of initial TCR-Cß, IL-2R, IL-1ß, and TNF-α mRNA in EMB were individually normalized to the corresponding keratin mRNA levels, which permitted more accurate comparison of these gene transcript levels. Cytokine mRNA expression was analysed only in EMB with keratin mRNA expression.

Statistical analysis

For qualitative cytokine analysis, data were analysed by Fisher's exact test for small sample sizes. For semiquantitative analysis, the levels of cytokine expression were compared by use of the Mann Whitney U test. P-values ≤0.05 were considered to be statistically significant.

RESULTS

Patient populations

The baseline characteristics of the studied patients are shown in table 1. The time of occurrence of the first acute rejection episode was not significantly different between steroid-resistant and steroid-sensitive patients ($p \geq 0.05$, Mann Whitney U test). Steroid-resistant patients had their first acute rejection at day 24 post-transplant (pTX, median: range 6 to 36) and steroid-sensitive patients at day 32 pTX (median: range 13 to 58). However, one year follow-up of these patients showed that rejection occurred in more patients in whom the first rejection episode was MP resistant and that in the same patient group the median number of rejection episodes was greater (p=0.04, Fisher's exact; p=0.01 Mann Whitney U, respectively; see table 1). During rejection only 2 of 16 patients were hemodynamically slightly compromised; one patient in whom the rejection proved to be MP sensitive and one patient with an MP resistant rejection showed signs of congestion. In both patients echocardiography showed thickening of the left ventricular walls and signs of impaired diastolic function. None of the 14 other tested patients had evidence of diastolic dysfunction during the rejection episode studied.

Table 1. Characteristics of the patient groups with a steroid-sensitive or steroid-resistant first rejection episode

Characteristic	Steroid sensitive	Steroid resistant	p.value
Patient (n)	7	9	
Age, years (mean)	45 (range 14-62)	45 (range 25-61)	NS
Gender (m/f)	7/0	8/1	NS
Primary disease			
Ischemic heart disease (n)	4	6	NS
Cardiomyopathy (n)	3	3	NS
Mismatch (mean ± SD)			
HLA-A	1.1 ± 0.7	1.3 ± 0.7	NS
HLA-B	1.7 ± 0.5	1.6 ± 0.5	NS
HLA-DR	1.4 ± 0.5	1.6 ± 0.5	NS
Day of first rejection (median)	32 (range 13-58)	24 (range 6-36)	NS
Patients with recurrence			
of rejection (n)	3	9	0.04[1]
Rejection incidence first year after			
transplantation (median)	1.8 (range 1-3)	3.7 (range 2-5)	0.01[1]

NS, Not Significant

[1] One patient who died within 3 month of transplantation of infection was excluded from the analysis

Intragraft mRNA expression in pre-treatment and post-treatment EMB specimens

For all EMB (n=30) tested the results of intragraft mRNA expression are summarized in table 2 for patients with successful anti-rejection therapy and in table 3 for patients with ongoing rejection in their control biopsy. Representative examples of qualitative RT-PCR analysis of intragraft mRNA expression in pre-treatment and post-treatment EMB are shown in figure 2.

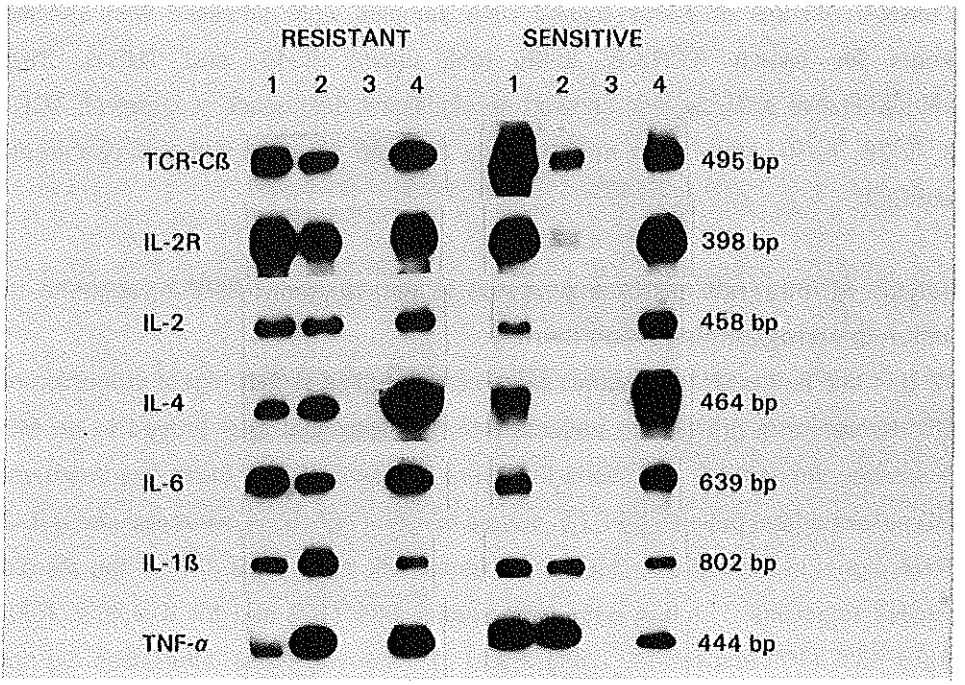

Figure 2. Southern blot analysis of RT-PCR amplified TCR-Cß, IL-2R (p55 chain), IL-2, IL-4 IL-6, IL-1ß, and TNF-α transcripts in EMB specimens before (lane 1) and after MP anti-rejection therapy (lane 2), negative H_2O control (lane 3), and in the positive control (PHA-M activated spleen cells; lane 4). Left, EMB specimens obtained from a patient with a steroid-resistant first rejection episode. Right, EMB specimens obtained a patient with a steroid-sensitive first rejection episode.

With the data unstratified for the efficacy of MP therapy, we detected no differences between the expression of cytokine mRNA profiles of pre-treatment or post-treatment EMB. In pre-treatment EMB we measured in 56% (9/16) IL-2 mRNA expression versus 50% (7/14) in the post-treatment EMB. We detected IL-4 in 31% (5/16) and 43% (6/14), respectively, and IL-6 in 56% (9/16) and 57% (8/14), respectively. Messengers coding for TCR-Cβ, IL-2R (p55 chain), IL-1ß, and TNF-α genes were also detectable in most of the post-treatment EMB (table 2 and 3). In an attempt to differentiate between mRNA expression levels of the persistently expressed genes, we performed semiquantitative RT-PCR analysis to estimate the relative initial amounts

of mRNA. Aliquots of 10-fold-diluted DNA samples were amplified with specific primers for keratin, TCR-Cβ, IL-2R (p55 chain), IL-1ß, and TNF-α. This semiquantitative analysis also showed no significant differences in intragraft TCR-Cβ, IL-2R, IL-1ß, and TNF-α mRNA levels between pre-treatment and post-treatment EMB.

Analysis of intragraft mRNA expression in pre-treatment EMB specimens in relation to steroid anti-rejection therapy

IL-2, IL-4 and IL-6 mRNA transcripts were expressed in a slightly higher proportion of EMB derived from grafts from patients with rejections that later proved to be MP irreversible compared to rejections that were MP sensitive. In these pre-treatment EMB obtained from patients that with steroid resistant rejections, we measured a IL-2 mRNA signal in 67% of the EMB (6/9) versus 43% of the EBB (3/7) in the steroid sensitive rejections. For IL-4 44% (4/9) versus 14% (1/7) and for IL-6 67% (6/9) versus 43% (3/7) respectively (table 2 and 3). However, these differences did not reach statistical significance and therefore overall pre-treatment mRNA profiles were not predictable for the efficacy of anti-rejection treatment with MP.

Analysis of intragraft mRNA expression in post-treatment control EMB specimens in relation to steroid anti-rejection therapy

When we analysed our results in relation to the efficacy of MP treatment as judged by the histological diagnosis of rejection in the control EMB, we found significant differences. After MP treatment, none (0/6) of the EMB taken from patients with MP-sensitive rejections expressed the IL-2 gene, in contrast to 88% (7/8) of the EMB obtained from patients with MP-resistant rejections (p=0.005, table 2 and 3). Moreover, intragraft IL-4 and IL-6 gene transcripts were hardly detectable (both 17%) in the MP reversible rejections, but in the ongoing rejections IL-4 mRNA transcription was found in 62% of the EMB (p=0.14), and IL-6 was detected in 88% of the EMB (p=0.03). In post-treatment EMB, TCR-Cβ, IL-2R, IL-1ß, and TNF-α mRNA transcripts were still detectable in most of the EMB (table 2 and 3), and consequently semiquantitative PCR analysis was performed.

Table 2. Messenger RNA expression of the constant region of the ß-chain of the TCR (TCR-Cβ), cytokines, and IL-2R (p55 chain) in EBB specimens before and after *successful* anti-rejection therapy with MP#, as detected by Southern blot analysis of polymerase chain reaction-amplified DNA

patient	TCR-Cβ	IL-2	IL-2R	IL-4	IL-1ß	IL-6	TNF-α
before treatment							
Ve	+	-	+	-	+	-	+
Ka	+	+	+	+	+	+	+
Ko	+	-	+	-	+	-	+
Vr	+	+	+	-	+	+	+
Fr	+	+	+	-	+	-	+
Ma	+	-	+	-	+	+	+
Pe	+	-	+	-	-	-	+
+/total	7/7	3/7	7/7	1/7	6/7	3/7	7/7
after treatment							
Ve	+	-	-	-	-	-	+
Ka	+	-	+	-	+	-	+
Ko	+	-	+	-	-	-	+
Vr	+	-	+	-	+	-	+
Fr	+	-	+	-	+	+	+
Ma	+	-	+	+	+	-	+
+/total	6/6	0/6	5/6	1/6	4/6	1/6	6/6

successful anti-rejection therapy with MP: before treatment, EMB specimens with infiltrates and myocyte damage, rejection grade 3: after treatment; EMB specimens with or without infiltrates but without myocyte damage, rejection grade 0 and 1 according to Billingham et al. (19).

-, no signal; +, positive signal

Table 3. Messenger RNA expression of the constant region of the ß-chain of the TCR (TCR-Cβ), cytokines, and IL2-R (p55 chain) in EMB specimens before and after *unsuccessful* anti-rejection therapy with MP#, as detected by Southern blot analysis of polymerase chain reaction-amplified DNA

patient	TCR-Cβ.	IL-2	IL-2R	IL-4	IL-1ß	IL-6	TNF-α
before treatment							
Ki	+	-	+	-	+	+	+
La	+	+	+	+	+	+	+
Be	+	-	+	-	+	-	+
Ze	+	-	+	-	-	-	+
Kn	+	+	+	+	+	+	+
Sc	+	+	+	-	+	-	+
Ro	+	+	+	+	+	+	+
Wa	+	+	+	+	+	+	+
So	+	+	+	-	+	+	+
+/total	9/9	6/9	9/9	4/9	8/9	6/9	9/9
after treatment							
Ki	+	+	+	+	+	+	+
La	+	+	+	-	+	+	+
Be	+	+	+	+	+	+	+
Ze	+	+	+	+	+	-	+
Kn	+	+	+	+	+	+	+
Sc	+	+	+	-	+	+	+
Ro	+	+	+	+	+	+	+
Wa	+	-	+	-	+	+	+
+/total	8/8	7/8	8/8	5/8	8/8	7/8	8/8

unsuccessful anti-rejection therapy with MP: before and after treatment; EMB specimens with infiltrates and myocyte damage, rejection grade 3, according to Billingham et al. (19).

-, no signal; +, positive signal

In control post-treatment EMB obtained from MP-reversible rejections we measured a significantly lower level of transcription for TCR-Cβ. mRNA compared to MP irreversible rejections (p=0.03; figure 3). Comparison of intragraft IL-2R, IL-1ß, and TNF-

α mRNA levels between MP-sensitive and -resistant rejections revealed significantly different mRNA levels in each instance (IL-2R, IL-1ß and TNF-α, p=0.03; figure 3).

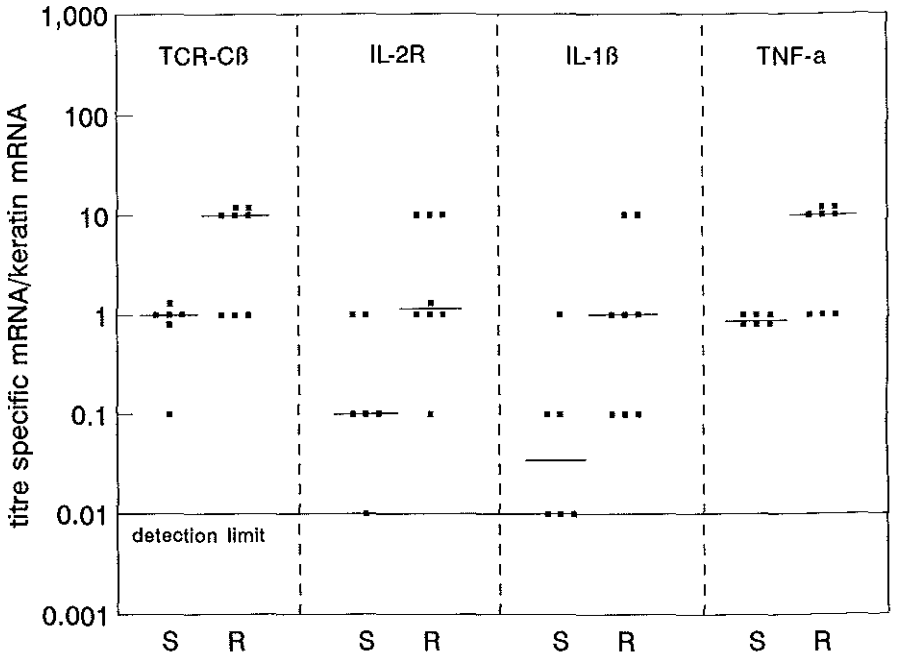

Figure 3. Titres were normalized for keratin mRNA expression. After MP anti-rejection therapy the corrected transcript levels for TCR-Cβ, IL-2R, IL-1ß, and TNF-α were significantly lower in EMB specimens of sensitive (S) rejections compared to MP resistant (R) rejections (p=0.03; Mann Whitney U test).

DISCUSSION

By the definition of persistent rejection after one course of MP, the overall incidence of resistance to steroid anti-rejection therapy in our heart transplant centre is 33% (3). One year follow-up of these patients showed that steroid-resistance was associated with recurrence of rejection later on. The development of steroid-resistant acute rejection was, however, not correlated with diastolic dysfunction of the transplanted heart. Clinically evident impaired graft function was only found in one of nine patients with a steroid-resistant first rejection episode.

In the rejection process, cytokines produced by allograft infiltrating cells mediate and

regulate the immune response towards the transplantated organ. In an attempt to clarify the mechanisms responsible for steroid resistance, we measured cytokine mRNA profiles in EMB during rejection, before and after MP therapy was given. As in our previous studies, we found that mRNA of the cytokines IL-2 and IL-6 are frequently expressed in transplanted hearts during acute rejection (15,16). These data suggest that cells transcribing the IL-2 gene or the IL-6 gene (or both) play a significant role in the rejection process. During acute allograft rejection a substantial number of both lymphocytes and monocytes infiltrate the allograft (19,21). It is reasonably to assume that a significant part of IL-6 mRNA expression is derived from infiltrating monocytes. However, results of the current retrospective study of mRNA profiles in EMB during rejection suggest that cytokine mRNA expression patterns prior to the first gift of anti-rejection therapy cannot predict the outcome of steroid anti-rejection therapy. More importantly, we did find that the efficacy of MP anti-rejection therapy is reflected by intragraft cytokine mRNA expression. Successful MP anti-rejection therapy resulted in an overall decrease of cytokine mRNA expression, but it was most prominent for IL-2 and IL-6. Intragraft mRNA expression in grafts with ongoing rejections remained upregulated or even increased. The inhibition of cytokine mRNA expression was accompanied by a significant decrease of TCR-Cβ mRNA expression, which confirms the histopathological analysis of the post-treatment control EMB.

There are various possibilities for GC to exert their immunosuppressive effect. *In vitro* studies showed that GC can specifically suppress transcription of the IL-2 gene of activated T-cells by blocking two transcriptional control sites: the nuclear factor of activated T-cells and activating protein-1, both present at the promotor site of the IL-2 gene (7,22,23). GC are also able to inhibit signal transduction through the IL-2R of activated T-cells which was shown by Paliogianni et al. in mitogen-stimulated peripheral blood mononuclear cells (24). GC may suppress T-cell activation through inhibition of the cytokine production by antigen presenting cells. In these cells GC bind to a specific intracellular steroid receptor, migrate to the nucleus at or near 5' flanking DNA of responsive genes, and directly inhibits cytokine gene transcription of the accessory cells (2,5). T-cells also have an intracellular steroid receptor, and consequently GC can directly affect T-cells through this pathway. After successful

MP treatment we found an overall downregulation of intragraft mRNA expression of immunoregulatory and inflammatory cytokines. In control post-treatment EMB, this was not observed in MP resistant rejections. An explanation could be a reduced GC receptor binding affinity of T-cells as has been described in association with a poor responses to systemic MP therapy in asthma (25). *In vitro* incubation of T-cells with the combination of IL-2 and IL-4 maintained glucocorticoid receptor binding affinity at a reduced level resulting in a diminished effect of MP on T-cell proliferation (17,25). In the current study, simultaneous intragraft IL-2 and IL-4 mRNA expression was only detectable in 1/7 (14%) of the EMB obtained from MP reversible rejections, in contrast to 4/9 (44%) EMB of the MP irreversible rejections. This suggests that also after clinical heart transplantation the combination of these cytokines might influence the glucocorticoid receptor binding affinity. Also interindividual variability of MP pharmokinetics has been reported (25,26,27). The occurrence of acute rejection was associated with short half-life of MP. This variability in pharmacokinetics could also contribute to a lack of immunosuppressive efficacy.

Immunoregulatory (IL-2) and inflammatory (IL-6) cytokine genes were associated with allograft rejection. Results of cytokine mRNA profiles in the pre-treatment EMB suggested that cytokine patterns cannot predict the outcome of MP anti-rejection therapy. After MP treatment, intragraft cytokine mRNA expression profiles were significantly different between MP reversible and ongoing rejection episodes. In contrast to the ongoing rejections, MP reversible rejection was associated with a significant inhibition of intragraft cytokine and TCR-Cβ mRNA expression. The *in vivo* observation that MP inhibits the transcription of both immunoregulatory and inflammatory cytokines within the transplanted organ confirms previous *in vitro* observations. We conclude that measuring intragraft cytokine mRNA profiles by the RT-PCR technique is an objective way to monitor the efficacy of MP anti-rejection therapy.

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Chapter 7

THE NATURE OF ACUTE REJECTION IS ASSOCIATED WITH DEVELOPMENT OF GRAFT VASCULAR DISEASE AFTER CLINICAL HEART TRANSPLANTATION

This chapter is based on articles published in:

J Heart Lung Transplant (in press)

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Transplant Proc 1997;29:2529-2530

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ABSTRACT

To determine mechanisms triggering graft vascular disease (GVD) after heart transplantation, we studied parameters reflecting both early and late intragraft allogeneic reactions. Using RT-PCR analysis, mRNA expression of IL-2, IL-4, IL-6, IL-10, IFN-γ, PDGF-α, and TGF-ß, was measured in endomyocardial biopsies (EMB) obtained from 34 recipients during the first acute rejection episode for rejectors (n=29) or at a comparable time post-transplant for non-rejectors (n=5), and at time of assessment of GVD by coronary angiography at one year (n=34). At time of assessment of GVD, mRNA expression of IL-2, IL-4, and IL-6 were hardly detectable, while messengers coding for IFN-γ, IL-10, TGF-ß, and PDGF-α genes were constitutively expressed. Moreover, intragraft mRNA patterns of cytokines and growth factors between patients with GVD (n=17) or without GVD (n=17) were comparable. In contrast, during the first acute rejection episode a completely different pattern was found. Development of GVD was associated with IL-2 mRNA expression and not with the other cytokines analysed. IL-2 mRNA was present in 77% of rejection EMB taken from patients with GVD versus 33% of the EMB from patients without GVD (p=0.03) and not detectable in EMB from non-rejectors. Also non-immunological risk factors such as longer ischemia time (median 193 vs 141 min; p=0.002) and higher donor age (median 32 vs 23 y; p=0.02) were associated with GVD. But no relation was found between these non-immunologic risk factors and IL-2 positive acute rejections. Non-specific risk factors and IL-2 positive rejections may independently trigger GVD after clinical heart transplantation.

INTRODUCTION

Long-term results of clinical organ transplantation are strongly influenced by graft failure due to chronic transplant dysfunction also known as chronic allograft rejection (1,2). After heart transplantation, the chronic rejection process is manifested by inflammation followed by accelerated allograft arteriosclerosis named graft vascular disease (GVD). This chronic process is characterized by concentric narrowing of arterioles i.e. intimal thickening with proliferation of smooth muscle cells. Another

feature is the irregular thickening of the donor coronary wall that affects the entire length of the vessel including the small branches (3). The latter feature makes GVD accessible to diagnostic procedures through EMB. The pathogenic mechanism that triggers and maintains this chronic process is subject of many studies. Both humoral and cellular alloimmunity may cause chronic allograft rejection (4). Moreover, multiple non-immunological risk factors may also contribute to the development of GVD (4). Potential non-immunological risk factors include prolonged ischemia time, reperfusion injury, cyclosporin dosage, transplant size, lipid abnormalities, age and gender of the donor. Potential immunological contributors are HLA incompatibility, CMV infection and disease, panel reactive antibody, and number and intensity of acute rejection episodes (4-7). During transplantation, ischemia and reperfusion damage cause injury to the endothelium of the transplanted heart (8). As a result of the non-specific injury activation of the complement and coagulation cascades takes place and damaged endothelial cells release various cytokines and growth factors (9-11). These soluble mediators (e.g. IFN-γ, tumor necrosis factor-α, IL-1, IL-6, PDGF-α) stimulate smooth muscle cell proliferation contributing to the intimal thickening that produces obstructive lesions (10-12). Moreover, cytokines and growth factors are potent activators of monocytes/macrophages and vascular endothelium upregulate expression of HLA antigens and adhesion molecules, and promote T-cell adhesion (10). The upregulated expression of HLA class I and II antigens on endothelial cells increases the immunogenicity of the graft, thereby promoting allogeneic responses. Several immunological factors influencing the induction of GVD have been found in experimental studies. These studies show that early immune responses to the allograft are associated with chronic allograft rejection later on. Firstly, Nakagawa and colleagues demonstrated a direct relation between acute rejection and GVD in the cardiac rabbit model (13). Secondly, in the Lewis-to-Fisher F344 rat cardiac allograft model, Russell et al. found significant mRNA expression of IFN-γ, monocyte chemoattractant protein-1 and IL-6 within the interstitium in the first weeks after transplantation (14). In the early period after transplantation, acute rejection is a severe immunogenic complication. A correlation between acute rejection and HLA matching has been extensively reported but the

effect of HLA matching on chronic rejection is controversial (15,16). Acute cellular rejection is attended with high intragraft production of cytokines (e.g. IL-2, IL-4, IL-6, IL-10, IFN-γ) and growth-factors (basic fibroblast growth factor) produced by infiltrating T-lymphocytes or macrophages (17-21). Libby et al. hypothesized that these cellular immune responses via the cytokine cascade trigger the development of GVD (22). However, despite numerous studies, the relation between acute rejection and the development of GVD after clinical heart transplantation remains controversial (6,7,23-25). Most of these studies related the number of acute rejection episodes with long-term graft survival but did not characterize these early allogeneic immune responses in more detail. Acute rejection episodes are the result of various immunological responses that can be identified by different cytokine patterns.

In the current study, we tried to associate these cytokine profiles accompanying acute rejections with the development of GVD. Therefore, intragraft mRNA expression of cytokines and growth factors involved in early and late immunogeneic immune responses were analysed. Using RT-PCR analysis, we measured mRNA expression of IL-2, IL-4 and IL-6 during acute rejection and at one year post-transplant in endomyocardial biopsies (EMB) obtained from patients with and without GVD. In addition, the role of IFN-γ, IL-10, PDGF-α, and TGF-ß in the process of GVD was studied. Contribution of other risk factors on the development of GVD after clinical heart transplantation was also analysed.

METHODS

Patients

We analysed mRNA expression of cytokines and growth factors in EMB obtained from 17 consecutive heart transplant patients with GVD lesions and 17 recipients (controls) without signs of GVD at one year after transplantation. Control patients were carefully matched for age, gender, date of transplantation (within 6 weeks), anti T-cell induction therapy, and original disease. Table 1 summarizes base-line characteristics of these patients at the time of transplantation. Patients were transplanted between November 1991 and August 1994 and received early prophylactic immunosuppression with BT563 (n=11), a murine anti-interleukin-2 receptor monoclonal antibody (CD25, Biotest Pharma, Dreieich, Germany) or with OKT3 (n=22; Ortho Pharmaceutical Corp, Raritan,

NJ) or with rabbit ATG (n=1). Maintenance immunosuppressive therapy consisted of cyclosporin A and low-dose steroids. Acute rejection was histologically diagnosed and scored by the pathologist P.E.Z. in EMB according to the criteria of the International Society for Heart and Lung Transplantation (26). EMB are taken weekly during the first 6 weeks, biweekly during the next month, monthly for the next four months and less often thereafter. Anti-rejection therapy was instituted in case of moderate rejection (multifocal aggressive infiltrates and/or myocyte damage, ISHLT grade ≥ 3A). GVD was defined as all vascular wall changes, including minor irregularities, assessed by visual analysis of coronary anglography at one year post-transplant and had been routinely interpreted by the cardiologist A.H.M.M.B. (6). For the present study, we analysed EMB taken from 34 patients at time of assessment of GVD at one year post-transplant and during their first acute rejection episode for the rejectors (n=29) or at a comparable period post-transplant for the non-rejectors (n=5).

RNA preparation and cDNA synthesis

For RT-PCR analysis, EMB were snap-frozen in liquid nitrogen and stored at -80°C. Messenger RNA extraction and transcription was performed as described previously (17). Briefly, total RNA was extracted from snap-frozen samples by a modification of the guanidinium method, as described by Chomczynski and Sacchi (27). EMB were homogenized in 500 µl 4 mol/L guanidinium-isothiocyanate in the presence of 20 µg poly A (Boehringer, Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1], respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 mol/L sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000xg at 4°C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand DNA synthesis was performed from 25 µl of the isolated RNA with 0.25 µg hexanucleotides (Promega Corporation, Madison, WI) and transcribed with 500 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture consisted of 10 µl 5x MMLV-RT buffer (250 mmol/L Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 µl dNTP (10 mM), 200 U of RNAsin (Promega) and 5 µl (0.1M) DTT.

PCR amplification and Southern blot analysis

Sequence specific primers (Clontech Laboratories, Palo Alto, CA) were used for amplification of the human cytokine genes. PCR primers detecting transcripts for the human house-keeping gene keratin were used as an internal control to monitor mRNA extraction and DNA amplification. For qualitative analysis, 5 µl DNA sample was added to 95 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler under the following

conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% PHA-M (Difco, Detroit, MI) for 4 h at 37°C. Messenger RNA from this positive control was extracted as described above. Negative controls consisted of omission of reverse transcriptase from the DNA synthesis reaction for each EMB followed by amplification in PCR with the complete range of cytokine primers, and the use of and diethylpyrocarbonate treated-H_2O as no-template reaction. After amplification, 16 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, UK) by electroblotting, and hybridized with $\gamma^{32}P$ labelled specific probes which are located across the splice-site. Hybridization was detected by autoradiography and indicated the presence of mRNA expression in the original biopsy. EMB taken during acute rejection were screened for IL-2, IL-4, and IL-6 mRNA expression and the one year EMB were analysed for cytokine mRNA expression of IL-2, IL-4, IL-6, IL-10, IFN-γ, TGF-ß and PDGF-α.

Competitive template RT-PCR for keratin and IL-10

To estimate the relative initial amount of functional IL-10 in EMB, a competitive template RT-PCR assay was used and comparison was made against the house-keeping keratin gene. Keratin is assumed to be expressed at a constant level in EMB (28). To obtain a standard curve for IL-10 and keratin, known amounts of internal control fragment were added in different dilutions to constant amounts of sample DNA for competitive co-amplification. The internal control was designed to generate a PCR product of a different size to allow differentiation between the amplified target and internal standard. Five and 10-fold dilutions of the keratin competitor template, ranging from 1 fg to 1000 fg in 5 µl, were co-amplified with 0.5 µl aliquots of sample DNA. Following PCR, using conditions as described previously, the amplification products were analysed by gel electrophoresis and the amount of products by the internal control and targets are determined for each individual reaction. The relative ethidium bromide intensity on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, NY). The logarithm of the ratio target/internal control is graphed as a function of the logarithm of the internal molar amount of the standard and at ratio 1, the starting concentration of keratin DNA prior to PCR is assumed to be equal to the known starting concentration the competing internal control.

Statistical analysis

The described immunologic and non-immunologic variables were selected, and the influence of these variables on the development of GVD was then assessed. For qualitative RT-PCR analysis, Fisher's exact test was used for small sample sizes. An unpaired Mann-Whitney U-test was used to define differences between continuous variables in subgroups. P. values ≤ 0.05 were considered to be statistically significant.

Table 1. Baseline characteristics of 34 heart transplant recipients.

	gender recipient	age (y)	heart disease	age donor (y)	ischemic time (min)	induction therapy
with GVD at 1-year						
1	M	65	IHD	26	210	OKT3
2	M	59	IHD	32	176	BT563
3	M	48	CMP	32	240	BT563
4	M	64	IHD	39	230	OKT3
5	M	61	IHD	25	196	BT563
6	M	56	IHD	20	147	BT563
7	M	51	IHD	21	147	BT563
8	F	62	IHD	35	210	OKT3
9	M	51	CMP	44	220	OKT3
10	M	60	CMP	35	170	OKT3
11	M	44	IHD	33	210	OKT3
12	M	59	IHD	42	230	BT563
13	F	57	CMP	31	149	OKT3
14	F	48	IHD	36	193	BT563
15	M	51	CMP	unknown	178	OKT3
16	M	58	IHD	24	138	ATG
17	M	55	IHD	42	127	OKT3
without GVD at 1-year						
1	M	59	CMP	26	110	OKT3
2	M	46	IHD	32	107	BT563
3	M	51	IHD	17	135	OKT3
4	M	60	IHD	41	142	OKT3
5	M	64	IHD	24	125	BT563
6	M	64	IHD	21	118	OKT3
7	M	64	IHD	37	196	BT563
8	M	61	IHD	21	160	OKT3
9	M	41	IHD	25	106	OKT3
10	F	45	CMP	20	210	OKT3
11	M	33	IHD	23	150	OKT3
12	M	25	CMP	23	120	OKT3
13	M	54	CMP	36	150	OKT3
14	F	34	CMP	28	141	BT563
15	M	58	IHD	15	152	OKT3
16	M	14	CMP	16	190	OKT3
17	M	61	CMP	23	140	OKT3

y, years; min, minutes; M, male; F, female; IHD, ischemic heart disease; CMP, cardiomyopathy

RESULTS

Intragraft mRNA expression at one year post-transplant

Messenger RNA expression of the house-keeping gene keratin was chosen as a marker for the total quantity of mRNA extracted. No statistical difference was found in the amount of reversely transcribed DNA for keratin between biopsies obtained from patients with GVD (n=17) and those without GVD (n=17) at one year. For patients with GVD the level of keratin mRNA expression varied from 5 to 500 fg and for patients without GVD from 5 to 200 fg (p=0.61 Mann Whitney U-test). This indicated that an equivalent of functional mRNA was isolated, which is corrected for reverse transcription and the efficacy of each reaction. We analysed EMB derived from heart transplant recipients (n=34) at 1-year post-transplant, for the expression of IL-2 and IL-4, predominantly expressed by infiltrating lymphocytes, and IFN-γ, IL-6, IL-10, TGF-ß and PDGF-α all produced by various cell types. A typical example of mRNA expression 1-year after transplantation is shown in figure 1.

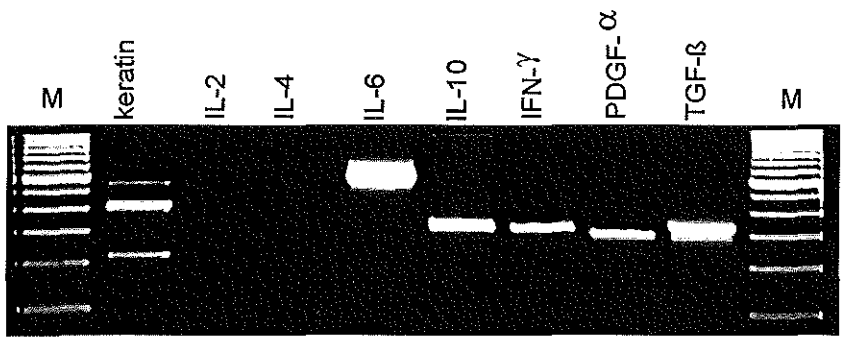

Figure 1. Expression of mRNA of keratin, cytokines and growth factors in an endomyocardial biopsy (EMB) obtained from a patient with graft vascular disease (GVD) at one year post-transplant. DNA was amplified by PCR and the products were electrophoresed on an agarose gel stained with ethidium bromide. In this EMB, PCR products of predicted size were observed for the house-keeping gene keratin, and for the cytokines IL-6, IL-10 and IFN-γ, and the growth-factors PDGF-α and TGF-ß.

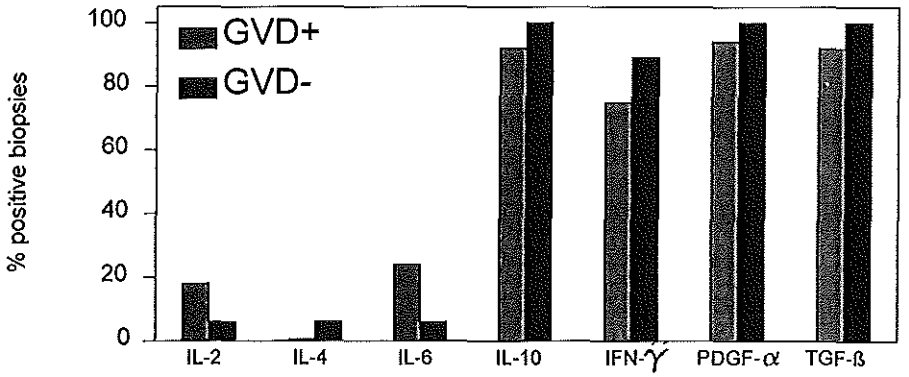

Figure 2. Analysis intragraft mRNA expression of the cytokines IL-2, IL-4, IL-6 and IL-10 and the growth factors, IFN-γ, PDGF-α, and TGF-ß at time of assessment of graft vascular disease (GVD) by coronary angiography at one year after heart transplantation. Data are expressed as the percentage of specimens containing mRNA coding for the above mentioned genes. No significant differences were found between patients with GVD and those without. None of the mediators analysed was discriminatory for GVD ($p>0.05$).

At time of assessment of GVD, we found that mRNA expression of IL-2, IL-4, and IL-6 were hardly detectable while messengers coding for IFN-γ, IL-10, TGF-ß, and PDGF-α genes were constitutively expressed. Intragraft mRNA expression of IL-2 was present in 12% (4/34), IL-4 in 3% (1/34), and IL-6 in 15% (5/34) of the EMB.
The intragraft mRNA profiles of the tested cytokines and growth-factors between patients with GVD (n=17) or without GVD (n=17) were comparable (figure 2). In addition, the level of IL-10 mRNA transcripts was comparable between both patient groups (figure 3). For patients with GVD the IL-10 (fg)/keratin (fg) ratio varied from 0.002 to 0.128 and for patients without GVD from 0.001 to 0.787 (p=0.76).

Intragraft mRNA expression during first AR episode

Early periods post-transplant (median 23, range 6-337 days post-transplant) from both rejectors and non-rejectors were studied and results were related to GVD later

on (table 2). From 4 out of 5 non-rejectors, EMB were analysed for the presence of IL-2, IL-4 and IL-6 mRNA expression. These EMB were collected between 24-32 days after transplantation. In these specimens neither IL-2 nor IL-4 mRNA expression was detectable but IL-6 mRNA transcripts were present in 3 out of 4 EMB tested. Twenty-nine out of 34 recipients were treated for acute rejection within the first year after transplantation (table 2). From 1 out of these 29 rejectors no first rejection EMB was available for analysis. We measured intragraft mRNA expression of IL-2, IL-4, and IL-6 in EMB (n=13) from patients with GVD and in EMB (n=15) from patients without GVD. During their first acute rejection episode, the intragraft cytokine mRNA profiles were significantly different between patients who were found to have or have no coronary GVD lesions at one year post-transplant. Analysis showed that development of GVD was associated with IL-2 mRNA expression during early acute rejection (figure 4). Intragraft IL-2 mRNA expression was present in 77% (10/13) of the EMB from patients with GVD at one year vs only 33% (5/15) of the EMB from patients without GVD (p=0.03). Development of GVD was not related to early intragraft mRNA expression of IL-4 or IL-6.

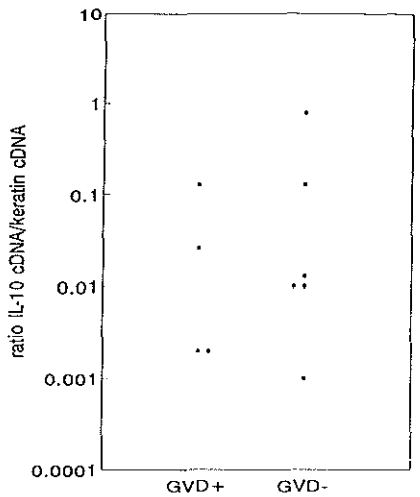

Figure 3. IL-10 cDNA / keratin cDNA ratio in endomyocardial biopsies (EMB) obtained from patients with graft vascular disease GVD (GVD+) or without GVD (GVD-) one year after heart transplantation.

Immunologic and non-immunologic risk factors

Patient demographics, stratified according to patients with and without GVD at 1-year are shown in table 1 and 2. Of the non-immunologic risk factors, both higher

donor age and longer ischemic time were positively correlated with the development of GVD at one year (table 1). Recipients with GVD were transplanted with older donor hearts (median 32 y, range 20-44 y vs median 23 y, range 15-41 y; p=0.02) and had longer ischemia time (median 193 min, range 127-240 min vs median 141 min, range 106-210 min; p=0.002). For patients with and without GVD the triglyceride levels were comparable: median 1.82 mmol/L, range 0.71-6.95 mmol/L and 2.13 mmol/L, range 1.03-4.25 mmol/L, respectively.

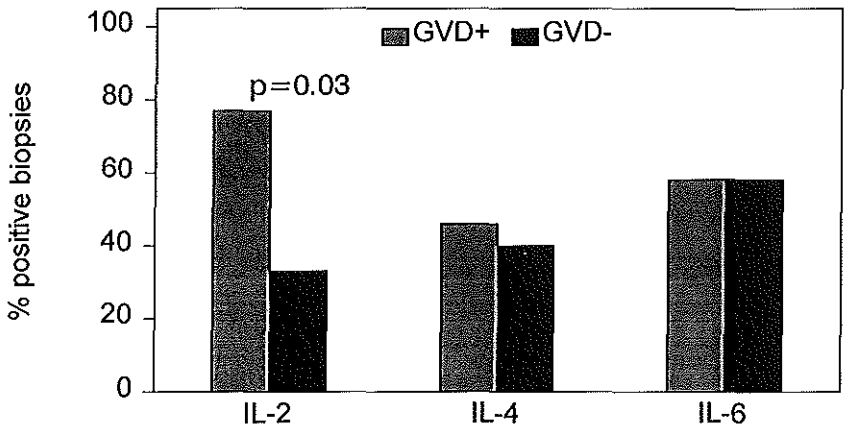

Figure 4. Cytokine mRNA analysis in rejection EMB from patients with (n=13) and without (n=15) angiographic evidence of graft vascular disease (GVD) at 1-year after heart transplantation. Data are expressed as the percentage of specimens containing mRNA for IL-2, IL-4 or IL-6. The proportion of rejection EMB expressing IL-2 mRNA was significantly higher in patients with GVD compared to patients without GVD (p=0.03, Fisher's exact). Development of GVD was not correlated with early intragraft mRNA expression of IL-4 and IL-6 (p>0.05).

However, none of the immunologic dependent risk factors analysed, including number of HLA mismatches, panel reactive antibody at time of transplantation, CMV serology of donor/acceptor, day of first rejection, and number of acute rejection

episodes during the first year after transplantation, was found to be associated with an increased risk of developing GVD (table 1 and 2).

Table 2. Immunologic risk factors for graft vascular disease (GVD) at 1-year.

patient	HLA MM (N)	PRA (%)	CMV serology d/r	first rejection (days)	acute rejection (N)
with GVD at 1-year					
1	6	0	-/-	-	0
2	4	0	-/-	19	1
3	6	0	-/+	-	0
4	5	0	-/-	30	3
5	5	0	-/+	7	3
6	6	0	-/+	6	3
7	5	0	-/-	19	3
8	4	0	+/+	-	0
9	5	0	+/+	67	5
10	5	3	+/+	34	1
11	4	19	+/-	24	4
12	5	4	-/+	59	1
13	2	5	+/-	29	5
14	3	0	+/-	7	4
15	5	ND	-/-	36	4
16	3	ND	-/+	36	3
17	4	0	+/-	31	4
without GVD at 1-year					
1	5	0	ND/+	60	1
2	4	0	+/+	7	4
3	4	0	-/+	-	0
4	4	0	-/+	337	1
5	5	3	-/+	16	4
6	5	0	ND/+	7	3
7	3	4	+/-	-	0
8	4	0	ND/+	24	3
9	4	5	+/-	23	3
10	6	0	+/+	23	1
11	5	0	-/+	24	4
12	4	4	-/-	16	5
13	4	90	-/-	57	1
14	6	15	+/-	6	5
15	3	0	-/+	23	2
16	6	0	-/-	13	2
17	6	0	+/+	17	4

GVD, graft vascular disease; MM, mismatches; PRA, panel reactive antibodies at time of transplantation; CMV, cytomegalovirus; N, number; d, donor; r, recipient; ND, not determined;

Relation between non-immunologic risk factors and intragraft IL-2 mRNA expression

The above described data show an association between intragraft IL-2 mRNA expression, longer ischemia time and higher donor age with development of early GVD. Nevertheless, it is evident that some data overlap between patients with and without GVD at one year. For instance, patients with short ischemia time can develop GVD at one year while intragraft IL-2 mRNA expression is not always found in patients with vascular lesions. Therefore, we analysed the interaction between non-immunologic risk factors and intragraft IL-2 mRNA expression (figure 5). In the IL-2 negative group, the data are clear, development of GVD was significantly associated with both prolonged ischemia time and higher donor age (A and C, $p<0.01$ and $p<0.05$, respectively). In the IL-2 positive group, this association is less pronounced. It seems that patients who received a transplant with relatively short ischemia time also develop GVD when the acute rejection is associated with intragraft IL-2 mRNA production (B). This suggests that non-immunologic risk factors and IL-2 positive acute rejections are independent risk factors involved in GVD development.

DISCUSSION

At our center, the angiographic incidence of GVD when even the smallest vessel wall irregularities are taken into account is 26 % at one year accumulating to 87 % at 8 years post-transplant. Accordingly, intima thickening is an ongoing process that eventually leads to complete obstruction of the coronary arteries resulting in graft dysfunction. Using the sensitive intravascular ultrasound technique, the prevalence of GVD was even higher (29). GVD can be detected not only in coronary vessels but also in the smaller intramyocardial vessels, present in EMB, reflecting a more generalised vascular lesion in the graft (3,30).

In the present study, we have examined the contribution of immunological and non-immunologic factors to the development of GVD after clinical heart transplantation.

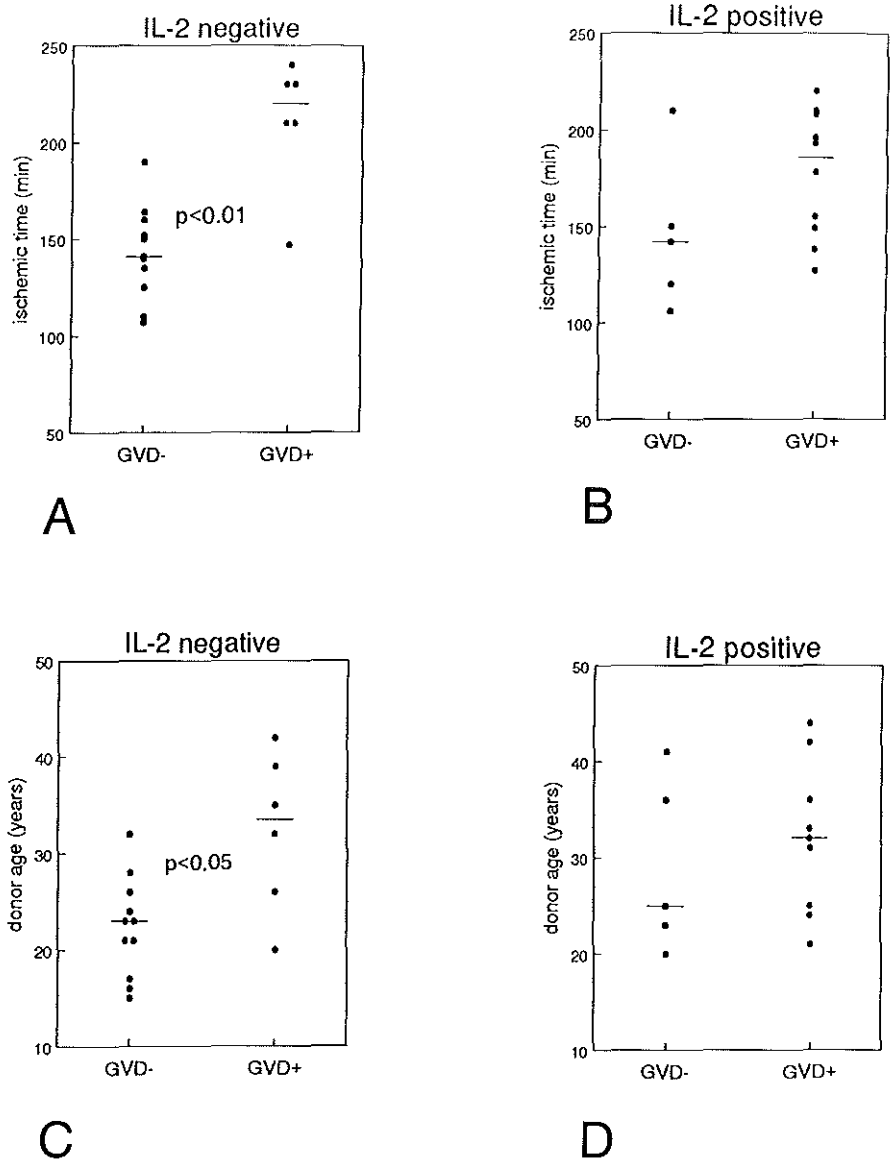

Figure 5. Analysis of the interaction between non-immunologic and immunologic factors in relation to development of early graft vascular disease (GVD). In the absence of intragraft IL-2 mRNA expression, both prolonged ischemic time (A) and higher donor age (C) were associated with development of GVD at one year ($p<0.01$ and $p<0.05$, respectively, Mann Whitney U test). In contrast, in the presence of intragraft IL-2 mRNA expression, no significant relation between ischemia time (B) and donor age (D) was found ($p>0.05$).

Analysis of 17 consecutive heart transplant recipients with GVD and 17 controls without GVD at one year, showed that both non immunological an immunological factors are associated with the development of GVD.

Older donor hearts, longer ischemia time and intragraft mRNA expression of IL-2 during the first acute rejection episode correlated with diagnosis of GVD at one year post-transplant. Recently, conflicting data were reported concerning the contribution of donor age on the development of GVD (6,7,25,29,31). In the study reported by Drinkwater et al., the 5 years incidence of GVD was comparable between patients who received hearts from donors older or younger than 45 years (31). Previously and in the present study, we found that development of GVD is associated with higher donor age (6). This is in line with data by Mehra et al (7), by Young et al (25) and by Rickenbacher et al (29). Apparently, older donor hearts are more vulnerable for factors that may initiate GVD. The ischemia time in patients with GVD was significantly longer compared to ischemia time in patients who remained free from GVD. It is known that ischemia causes damage to the endothelium of the graft (8). Yilmaz et al. clearly showed, in rat renal allografts, that prolonged ischemia time resulted in vascular arteriosclerosis (32).The response to injury hypothesis proposed that injury to the endothelium is the initiating event in atherosclerosis (33,34). Damaged endothelial cells release basic fibroblast growth factor, macrophage chemoattractant protein-1, IFN-γ, IL-6 and PDGF (10,22,35) These multifunctional mediators contribute to smooth muscle cell proliferation. Shortly after transplantation, infiltrating macrophages are seen in both allo- and isografts, reflecting a general, non-immunological repair mechanism (36). In allografts upregulation of macrophage chemoattractant protein-1, IFN-γ, and IL-6 mRNA transcripts is associated with macrophage activation (14). Macrophage chemoattractant protein-1 induces cellular infiltration into inflammatory sites. Characteristic for GVD after heart transplantation is the selective involvement of the engrafted heart arteries with sparing of the host's native vessels, indicating that immunological mechanisms are involved in the pathogenesis of GVD (3). Repetitive endothelium injury by immunologically mediated responses contributes to the development of GVD (37). IFN-γ secreted by the damaged endothelial cells, as a

result of ischemia, increases the expression of adhesion molecules and HLA antigens on endothelial cells. These cells are then targets for immune mediated injury, thereby promoting cellular allograft rejection. Furthermore, histocompatibility differences between donor and recipient influence the incidence of acute rejections (15,38), which in turn, are often associated with accelerated development of GVD (23,24). In the current study, the number of HLA mismatches, the day of first rejection and the number of acute rejection episodes were not correlated with development of GVD at one year. In contrast to transplantation of other organs, the diagnosis of acute rejection after clinical heart transplantation is solely based on routine histology and not on graft function. In liver transplantation, histological signs of acute rejection may be accompanied by a deteriorating as well as with a improving graft function (39). This discrepancy is reflected in the intragraft mRNA cytokine pattern found. Liver graft dysfunction is associated with intragraft IL-2 mRNA expression while down-regulation of this immune response is mediated by locally produced IL-4. Also after heart transplantation the infiltrating cells may secrete different cytokines that determine the nature and the intensity of the rejection. To identify these factors we evaluated mRNA expression of IL-2, IL-4 and IL-6 in EMB obtained from rejectors during their first acute rejection episode and in EMB from non-rejectors taken at comparable period post-transplant. Only intragraft IL-2 mRNA expression correlated with GVD ($p=0.03$, figure 4). Intragraft IL-2 mRNA transcripts were not detectable in EMB obtained from the non-rejectors one-month after transplantation. Recently, we also reported that *in vitro* production of IL-2 and IFN-γ by graft infiltrating T-lymphocytes during the first 6 months after transplantation correlated with GVD at one year (40). Also Young et al. suggested a relation between the IL-2/IL-2receptor pathway and development of GVD (41). The prominent role for IL-2 and IFN-γ in the pathogenesis of GVD was already hypothesized by Libby et al. who postulated that IL-2 and IFN-γ might trigger a wave of macrophage-derived cytokines that subsequently could induce smooth muscle cell proliferation (22). In our patient population, additional analysis showed no relation between prolonged ischemia time or older donor hearts and intragraft IL-2 mRNA expression (figure 5). These results suggests that non-immune risk factors

and IL-2 positivity are two independent mechanisms both leading to early GVD. But it does not exclude the possibility that in individual patients endothelium damage by these non-specific factors induce intragraft IL-2 mRNA expression which in turn triggers GVD. Nevertheless, also patients without acute rejections will develop GVD which indicates that other factors such as humoral alloimmunity might be involved in the pathogenesis of this disease as well (42). At time of assessment of GVD by coronary angiography at one year, the intragraft mRNA profile of cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ) and growth factors (TGF-ß, PDGF-α), and also the level of cytokine mRNA expression (e.g. IL-10) was comparable between patients with and without GVD. Apparently events prior to the diagnosis of GVD play a role in its pathogenesis. In the early period after transplantation, grafts experience endothelial injury as a consequence of ischemic damage and allogeneic reactions. This is associated with an increased local production of various cytokines and growth factors, the pattern of which may determine the rapidity of the progression to GVD, a complication eventually affecting almost all heart transplant patients.

In conclusion, this study links early non-immunologic and immunologic events with accelerated development of GVD. Our data show that prolonged ischemia time, higher donor age and IL-2 positive allogeneic reactions are associated with early development of GVD after clinical heart transplantation.

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Chapter 8

INTRAGRAFT IL-4 mRNA EXPRESSION IS ASSOCIATED WITH DOWN-REGULATION OF LIVER GRAFT REJECTION

This chapter is published in:

Clin Transplantation 1996;10:542-549

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ABSTRACT

The mechanism underlying spontaneously resolving allograft rejection following clinical liver transplantation is unidentified. In this process, immunoregulatory T helper (Th)-2 cytokines like IL-4, often identified with down-regulation of the Th1 dependent (IL-2) cell mediated response, might play a significant but unknown role. For this reason, we analysed mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) in 57 biopsies derived from 19 recipients. Specimens included biopsies without evidence of rejection (n=36), biopsies with histological evidence of rejection (n=10) not followed by clinical signs of graft rejection, and biopsies with histological rejection that were accompanied with clinical rejection (n=11), defined by rising serum bilirubin and aspartate amino transaminase (ASAT) levels. Intragraft IL-4 mRNA expression significantly correlated with spontaneously resolving rejections. In 70% (7/10) of these biopsies, IL-4 mRNA was detectable while only 19% (7/36) of the biopsies without signs of rejection ($p<0.01$; Fisher's exact test) and 18% (2/11) of the rejection biopsies concurrent with graft dysfunction expressed the IL-4 gene ($p=0.03$). In contrast, IL-2 mRNA expression was not detectable in biopsies derived from the spontaneously resolving rejections. None (0/10) of these samples expressed the IL-2 gene which was not significantly different from the proportion of biopsies transcribing the IL-2 gene in the absence of rejection (11%, 4/36). IL-2 mRNA expression was found more often in biopsies associated with graft dysfunction (36%, 4/11). These results show that IL-4, in contrast to IL-2 mRNA expression, is associated with spontaneously resolving liver rejection. This suggests that Th2 cells down-regulate the Th1 dependent cell mediated immune response after clinical liver transplantation.

INTRODUCTION

Acute cellular rejection is a common phenomenon after liver transplantation (1-5). The histological hallmark of acute cellular rejection is a combination of three characteristic features: (1) predominantly mononuclear portal inflammation; (2) bile duct inflammation and/or damage; and (3) subendothelial localization of

mononuclear cells in the portal and central veins (2,4). In most cases, these histological signs of rejection are associated with clinical signs of rejection, e.g. changes in the amount and colour of bile together with biochemical evidence of deteriorating liver functions (1-3). However, from routinely performed biopsies it is known that histologic signs of rejection not always correlate with a deranged liver function, necessitating additional immunosuppression (1-3). The cellular infiltration observed during the first weeks after transplantation may in some instances be self-limiting and has no implication for ongoing or future graft damage (3-5). Characterization of these spontaneously resolving mononuclear cellular infiltrates could learn us more on the immunological processes leading to graft acceptance and would also be helpful in deciding when to refrain from or to initiate anti-rejection therapy. The rejection process is caused by a population of T cells that infiltrate the transplanted organ. The interaction between infiltrated activated cells and the allograft is mediated and regulated by locally produced cytokines. The graft can be infiltrated with functionally distinct subsets of T helper cells, termed Th1 and Th2, defined by their cytokine repertoire (6-8). Th1 cells produce e.g. IL-2, IFN-γ, TNF-α, TNF-ß, whereas Th2 cells produce e.g. IL-4, IL-5, IL-6, IL-10. After transplantation, the occurrence of graft rejection is primarily associated with increased production of Th1 cytokines (e.g. IL-2, IFN-γ) while Th2 (e.g. IL-4, IL-10) cytokines may suppress allograft rejection (9-11). These Th2 cytokines down-regulate the cell mediated immune responses by inhibition of the production of Th1 cytokines (12-14). Therefore, it could be envisaged that also after clinical organ transplantation, locally released Th2 cytokines could prevent graft destruction by ameliorating allogeneic reactions. Detection of Th2 cytokines in grafts with spontaneously resolving histological signs of rejection would be compatible with this hypothesis.

Several clinical studies have analysed the intragraft cytokine mRNA patterns (15-17) or cytokine protein production (18,19) in liver biopsies. However, these reports were mainly focused on histological features of acute liver rejection, irrespective of the clinical outcome. In order to further understand the mechanisms involved in the so-called self-limiting liver graft rejection, we analysed expression of IL-2 (Th1) and IL-4 (Th2) mRNA, using the RT-PCR technique, within liver allografts showing

histological signs of rejection with and without clinical signs of rejection.

MATERIALS AND METHODS

Patient specimens

Archival post-transplantation liver biopsy specimens (3-512 days post-transplant) from 19 patients (7 males and 12 females) transplanted between 1991 and 1993 were selected. From these patients one or more biopsies were available for RT-PCR analysis. The primary indications for liver transplantation were acute liver failure (n=7), cholestatic liver disease (n=7), posthepatic liver disease (n=2), post alcoholic liver disease (n=2), cryptogenic liver disease (n=1). Graft biopsies were taken according protocol or on indication in case of deteriorating liver function (table 1 n=21, and table 2 n=37). Immediately following procurement, a portion of each biopsy was snap-frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. The biopsy specimens were processed in standard fashion, stained with haematoxylin and eosin, periodic acid-Schiff, and iron and had been routinely interpreted by the pathologist P.E.Z. For the present study, the biopsy specimens were reviewed by P.E.Z. and H.J.M. both blinded to clinical information. All samples were obtained from patients who had received a HLA-mismatched cadaver donor liver. Standard immunosuppression consisted of azathioprine, cyclosporin A and low-dose methylprednisolone. Biliary reconstruction was performed with a T-tube and bile was inspected daily to assess its quantity and quality.

Management of rejection

A clinical diagnosis of rejection was suspected and considered likely if: (1) there was an increase in bilirubin and/or transaminases in successive days; (2) there was a decrease in the amount and colour of bile; and (3) other events were excluded radiologically, microbiologically or virologically. Cellular rejection was reported according to the Snover criteria [2]. In brief, grade 0, lymphocytic or mixed portal infiltrate, < 50% damaged bile ducts, no endothelialitis; grade 1, as above, with endothelialitis; grade 2, lymphocytic or mixed portal infiltrate, > 50% damaged bile ducts, with or without endothelialitis; grade 3, acute rejection plus arteritis, paucity of bile ducts, or central hepatocellular ballooning with confluent dropout of hepatocytes. Treatment for acute cellular rejection was given if the clinical diagnosis was considered to be probably correct and if there was histological evidence of cellular rejection. Patients received methylprednisolone for treatment of acute rejection at a dosage of 1000 mg/day for 3 consecutive days and OKT-3 monoclonal antibodies for steroid-resistant or severe rejection.

mRNA preparation and cDNA synthesis

Messenger RNA extraction and transcription was performed as described previously (20). Briefly, we

used a modification of the guanidinium method as described by Chomzynski and Sacchi (21). Total RNA was extracted from snap-frozen samples. Liver tissue was homogenized in 500 µl 4 M guanidine thiocyanate in the presence of carrier mouse 3T3 cells (ATCC, Rockville, MD) as described before (20). These carrier cells are not recognized by the primers used in the RT-PCR. The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1] respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000xg at 4°C and washed with 500 µl ice-cold (-80°C) 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from 25 µl of the isolated RNA with 0.25 µg hexanucleotides (0.5 µg/µl; Promega Corporation, Madison, WI) and transcribed with 2.5 µl Moloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µl; Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture contained 10 µl 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 µl dNTP (10 mM) 0.5 µl of RNAsin (400 U/µl; Promega) and 5 µl DTT (0.1M).

PCR amplification and Southern blot analysis

Sequence specific primers (Clontech Laboratories, Palo Alto, CA) were used for amplification of the human cytokine genes. PCR primers detecting transcripts for the human keratin gene were used as an internal control to confirm successful RNA extraction and cDNA amplification (20). All primers were located next to splice sites to be able to discriminate for mRNA only. For qualitative analysis, five µl cDNA sample was added to 95 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. For semi-quantitative analysis, cDNA samples were titrated (ten-fold) and aliquots of each dilution were amplified. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler (Biomed-60, Germany) using the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. As positive control for the cytokines and keratin we used cDNA isolated from 10^6 Hut78 (ATCC; a human T cell-line with constitutive cytokine mRNA transcription). After amplification, 16 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, England) by electroblotting and hybridized with $\gamma^{32}P$ labelled specific probes which are located across the splice-site. Hybridization was detected by autoradiography and indicated the presence of mRNA expression in the original biopsy. To estimate the relative initial amount of target cDNA, aliquots of titrated cDNA samples were amplified using specific primers for keratin. After titration, the relative amount of target cDNA was determined by the highest dilution showing positive signal. Sequential biopsies from individual patients

were isolated at the same time and the titre of keratin cDNA in all liver biopsies varied not more then one dilution step. There was no statistically significant difference in the amount of reversely transcribed cDNA for keratin between biopsies with Snover rejection grade >1 (n=21) and those with Snover rejection grade ≤1 (n=37; p= 0.57 Mann-Whitney-U test). To exclude inter and intra-assay variability, amplification of titrated cDNA of the HuT78 cell-line was included in each analysis. The expression of mRNA for keratin in separately isolated control cell-line was highly reproducible as inter and intra assay variation did not exceed a single dilution step. In the present study, only biopsies with significant keratin gene expression were analysed.

RESULTS

In total 58 biopsies derived from 19 liver transplant recipients were analysed. We observed histological signs of rejection, i.e. Snover rejection grade >1, in 21 biopsies (table 1). In 11/21 biopsies this histological rejection corresponded with clinical signs of rejection with a rise in median serum bilirubin from 142 µmol/l (range 39-225 µmol/l) to 178 µmol/l (range 35-318 µmol/l) and median ASAT levels from 58 U/l (range 12-234 U/l) to 90 U/l (range 14-230 U/l). This group therefore received anti-rejection therapy. The remaining 10/21 biopsies with Snover >1 were not accompanied by a clinical diagnosis of rejection. Median serum bilirubin in this group decreased from 119 µmol/l (range 28-276 µmol/l) to 111 µmol/l (range 32-310 µmol/l) and median ASAT levels from 35 U/l (range 14-133 U/l) to 30 U/l (range 14-90 U/l). This group did not receive anti-rejection therapy and subsequently showed no clinical or histological signs of rejection in the following 2 weeks (except patient Ra). The 37 biopsies with Snover ≤1 (table 2) corresponded with median serum bilirubin changes from 48 µmol/l (range 9-289 µmol/l) to 42 µmol/l (range 8-288 µmol/l) and median ASAT levels from 30 U/l (range 8-423 U/l) to 32 U/l (range 8-175 U/l). In this group, Snover ≤1, anti-rejection therapy was given in one instance. In this biopsy histology did not correspond with deranged graft function. Histology of the Snover >1 biopsies was not different between biopsies obtained from patients with or without clinical signs of rejection. Respectively 6/10 biopsies of the spontaneously resolving rejections and 7/11 biopsies of the clinical rejections had Snover rejection grade ≥ 2.

Table 1. Sample profile and cytokine mRNA expression in liver biopsies with histological evidence of rejection (Snover >1) not followed by clinical signs of graft rejection and biopsies with histological evidence of rejection accompanied with clinical significant rejection, defined by rising serum bilirubin and ASAT levels.

biopsy[a]	days pTX[b]	IL-2[c]	IL-4	control/indication or protocol biopsy
without clinical rejection				
Wi1	13	-	+	control[d]
Wi2	20	-	+	protocol/HBV[e]
Me1	9	-	-	control[d]
Me2	14	-	-	protocol/HBV[e]
My1	7	-	+	protocol
Ve3	24	-	+	protocol
De1	7	-	-	protocol
He1	7	-	+	protocol
Ti1	26	-	+	indication/cholestasis e.c.i
Ra2	12	-	+	control[d]
with clinical rejection				
La1	7	-	-	protocol
La5[f]	207	-	-	indication
La6	213	-	-	control[d]/ongoing rejection
La7	215	+	-	indication
Ke2	13	-	-	indication
Ra1	6	-	+	protocol
Ra3	19	-	-	indication
Mi1	17	+	-	indication
Re2	7	+	-	protocol
Kr3	24	-	-	protocol
Fo1	6	+	+	indication/ongoing rejection

[a] biopsy description; composed of patient identification and biopsy number analysed

[b] days post-transplant (pTX)

[c] - no signal, + positive signal after hybridization

[d] biopsies taken after anti-rejection therapy with high doses methylprednisolone, biopsies were taken 2-4 days after last gift

[e] screening re-infection HBV

[f] patient self-weaned his cyclosporin A medication

Table 2. Sample profile and cytokine mRNA expression in liver biopsies without histological evidence of rejection (Snover ≤1) and without clinical significant rejection

biopsy[a]	days pTX[b]	IL-2[c]	IL-4	control/indication or protocol biopsy
Wi3	206	-	-	protocol/HBV[e]
Wi4	355	-	-	protocol/HBV[e]
Me3	21	-	-	protocol/HBV[e]
Me4	357	-	-	protocol/HBV[e]
My2	20	-	-	protocol
My3	97	-	-	indication[f]
Ve1	9	-	-	protocol
Ve2	17	-	+	protocol
Ve4	31	-	+	indication[f]
La2	17	-	-	control[d]
La3	25	-	+	protocol
La4	95	-	-	protocol
Ke1	6	-	-	protocol
Ke3	18	-	-	control[d]
Mi2	41	+	-	indication[f]/CMV
Re1	3	+	-	indication/preservation injury
Re3	38	-	-	indication/preservation injury
Kr1	7	-	-	protocol
Kr2[g]	22	+	+	indication[f]; rejection
Kr4	36	-	-	indication[f]
Kr5	104	-	-	indication[f]
Fo2	191	-	-	indication[f]
Fo3	405	-	-	indication[f]
Bu1	23	-	+	protocol/HBV[e]
Bu2	87	-	-	protocol/HBV[e]
Bu3	358	-	-	protocol/HBV[e]
Ka1	9	-	+	protocol/HBV[e]
Ka2	15	-	-	protocol/HBV[e]
Ka3	22	-	+	protocol/HBV[e]
Ka4	43	-	-	protocol/HBV[e]
Ka5	95	-	-	protocol/HBV[e]

Ka6	512	-	-	protocol/HBV[e]
Ku1	6	-	-	protocol
Ku2	20	-	-	protocol
Ja1	98	-	-	indication[f]/CMV
Br1	40	+	+	indication[f]
Br2	50	+	-	indication[f]

[a] biopsy description; composed of patient identification and biopsy number analysed

[b] days post-transplant (pTX)

[c] - no signal, + positive signal after hybridization

[d] biopsies taken after anti-rejection therapy with high doses methylprednisolone, biopsies were taken 2-4 days after last gift

[e] screening re-infection HBV

[f] biopsies were taken in case of deteriorating liver functions

[g] patient received anti-rejection therapy with high doses of methylprednisolone

Alterations in mRNA expression profile were analysed in relation to histology (Snover ≤1 vs Snover >1), clinical criteria for diagnosis of rejection (treated rejections), and spontaneously resolving rejections (Snover >1 without graft dysfunction)

Results of sequential liver biopsies are presented in table 1 (Snover >1) and table 2 (Snover ≤1). Cytokine mRNA analysis showed that IL-2 gene expression was comparable when rejection was diagnosed on histological grounds (19%, 4/21 vs 14%, 5/37) whereas IL-2 gene expression was seen more frequently when rejection was based on clinical grounds (42%, 5/12 vs 9%, 4/46). Interestingly, IL-2 mRNA expression correlated better with clinical than with histological criteria of rejection. IL-4 gene expression also correlated with rejection, but only when histology was used for rejection diagnosis. IL-4 mRNA was detected in 43% (9/21) of the biopsies with Snover >1 classification and in 22% (8/37) of the Snover ≤1 classified biopsies. In contrast, when clinical criteria were used for rejection diagnosis, a totally different pattern emerged. IL-4 mRNA transcripts were detectable in 25% (3/12) of the biopsies that were followed by anti-rejection therapy vs 30% (14/46) of the biopsies from non-treated patients.

In the group without histological evidence of rejection (Snover ≤1) as classified in the review study, anti-rejection therapy had been given in one patient, this biopsy was not included in the present study. Representative examples of intragraft cytokine mRNA expression of sequentially taken liver biopsies from a patient with a

spontaneously resolving rejection episode and from a patient with a clinical rejection episode before and after anti-rejection treatment are shown in figure 1A and 1B respectively. Additional analysis showed that the detection of IL-4 mRNA correlated with uncompromised graft function in spite of concomitant histological signs of rejection (figure 2). Significantly more of these biopsies expressed IL-4 mRNA (70%, 7/10) compared to biopsies without any evidence of rejection (19%, 7/36; $p<0.01$, Fisher's exact test) or to biopsies with rejection in combination with clinical signs of rejection (18%, 2/11; $p=0.03$). In contrast, IL-2 mRNA expression was not detectable in biopsies with spontaneously resolving rejection. None (0/10) of these biopsies expressed the IL-2 gene which was not significantly different from the proportion of biopsies that expressed IL-2 in the absence of rejection 11% (4/36) whereas 36% (4/11) of the biopsies derived from the treated patients expressed IL-2 mRNA (figure 2).

Cytokine mRNA profile during rejection

From 5 patients (Wi, Me, La, Ra, Kr) multiple biopsies were analysed. We found in 2/5 patients that the intragraft cytokine mRNA profile changed during one rejection episode (patient La and Kr). These two patients had an ongoing rejection episode and consequentially samples were measured after treatment with methylprednisolone (table 1 and 2). The cytokine mRNA profile of patient Ra changed from IL-4 positive (Ra2) to IL-4 not detectable (Ra3) when histological rejection was accompanied with clinical rejection (table 1).

DISCUSSION

It has been reported by several groups that despite histological evidence of acute rejection in liver biopsies, treatment for rejection was not always necessary (1-3). In these instances patients had no associated liver dysfunction. However, a retrospective review of 8 liver biopsies specimens by Dousset et al (5) showed that it is even possible that patients with concomitant significant biochemical changes were able to resolve their allograft rejection without additional immunosuppression. Nevertheless, none of these studies could demonstrate a particular feature that could recognize or predict these spontaneous reversible rejections.

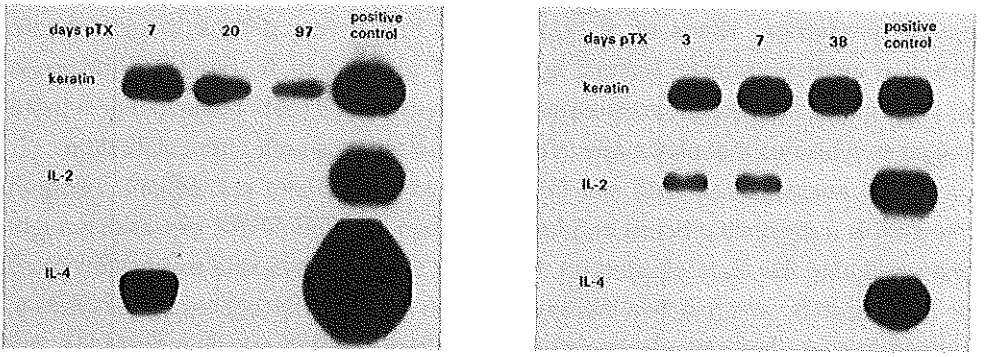

Figure 1. Southern blot analysis of RT-PCR amplified keratin, IL-2 and IL-4 transcripts from liver biopsies and positive control cell-line (HuT78 cells; a human T cell line with constitutive cytokine mRNA transcription). Keratin was used as a positive control for amplification. (left) Biopsies obtained from patient My taken at day 7 post-transplant (pTX), histological rejection spontaneously resolving; day 20 pTX, no evidence of rejection; day 97, no evidence of rejection. (right) Biopsies obtained from patient Re taken at day 3 pTX, no evidence of rejection; day 7 pTX, histological and clinical rejection, anti-rejection therapy given; day 38 pTX, no evidence of rejection.

Analysis of the immune response in the rat showed that not only animals that reject their liver allograft but also those that eventually accept their transplant undergo tissue damage (22). In the rejecting animals graft loss was accompanied by increased biochemical markers of cholestasis. In the present study, we found that tissue damage was accompanied by graft dysfunction in half of the cases only, while in the remaining 50% no biochemical signs of rejection were observed. This suggests that also after clinical liver transplantation tissue damage is part of a normal allogeneic response within the graft which can also be detected in the absence of graft dysfunction. This observation raises the question whether the infiltrates histologically observed in the absence of clinical rejection represent cells suppressing the allogeneic response. Cytokines regulate the immune response both during rejection episodes and during immunological quiescence. The same cytokine may, in these different situations exert either stimulatory or inhibitory effects. Still, monitoring of cytokines thought to be involved in rejection processes (Th1; IL-2) or to

be associated with maintenance of the allograft (Th2; IL-4), might provide insight in the complexity of the immune response after liver transplantation. In an attempt to correlate clinical complications after liver transplantation with systemic cytokine protein production, Kraus et al (23) showed that it was difficult to differentiate between graft dysfunction caused by allograft rejection or by other complications such as infections. Therefore, analysis of locally activated cytokines might give more insight in cytokines that play a role in the immune response after transplantation.

We have analysed our data per individual biopsy and not per patient. Allograft rejection is a dynamic process and for this reason cytokine mRNA expression might change during the state of rejection. Moreover, intragraft cytokine expression could be influenced by anti-rejection therapy with high doses of corticosteroids. Therefore, biopsies taken after anti-rejection treatment during ongoing rejection episodes, are not comparable with specimens obtained before anti-rejection therapy. From *in vitro* studies there is evidence that corticosteroids inhibit cytokine mRNA transcription (24). However, *in vivo* the effects of anti-rejection therapy on the transcriptional level of these cytokines within the transplanted organ is unknown. In the qualitative study presented here, intragraft IL-2 mRNA expression was positively correlated with clinical signs of rejection and not with histological signs of rejection. In addition, IL-2 mRNA expression was not detectable in grafts with spontaneously resolving rejections. Still, a considerable number of rejecting livers also did not express IL-2 transcripts during the effector phase which suggests that the presence of IL-2 is not always required for acute cellular rejection (15). The proliferation of graft infiltrating IL-2 negative cells might be regulated by other cytokines than IL-2. IL-2 knockout mice reject their pancreatic islet cell allografts in the presence of IL-4, IL-7 (a T-cell growth factor) and IFN-γ mRNA transcripts (25). However, it is known that expression of the IL-2 gene is an early but transient activation marker with a brief spike expression after activation and therefore present during the early phase of development of rejection (26-28). Therefore, it could be assumed that biopsies already had expressed IL-2 before the overt manifestation of clinical and/or histological rejection. After clinical kidney transplantation, Dallman et al. (26) found that in patients in whom rejection was confirmed histologically, intragraft IL-2 gene expression preceded the clinical onset of rejection.

After clinical liver transplantation we found that the intragraft cytokine mRNA profiles between spontaneously resolving rejections and those necessitating anti-rejection therapy were significantly different. In rodents, results of intragraft mRNA expression

were conflicting for liver allografts that were spontaneously tolerated or rejected in the absence of immunosuppression. Farges et al (29) showed that accepted allografts had lower intragraft IL-4 mRNA levels compared to rejected allografts while others found similar levels (30,31). However, long-term non-responsiveness in cyclosporin A treated rejectors was associated with significant IL-4 mRNA levels transcribed by graft infiltrating cells after in vitro allogeneic stimulation (32). In the present study, the Th2 cytokine IL-4 was more frequently expressed in biopsies with histological proved rejection (Snover > 1 classification) indicating that infiltrated cells are responsible for the IL-4 gene expression. In contrast, IL-4 mRNA expression was not associated with clinical criteria of rejection. Most interestingly, biopsies from patients with stable biochemistry accompanied by histological signs of rejection expressed significantly more often IL-4 mRNA than biopsies from patients with deteriorating graft function concomitant with similar histology. This might implicate that functionally different cell populations, i.e. Th1 and Th2 cells, have both infiltrated the allograft. A higher frequency of intragraft IL-4 mRNA expression in liver allografts with histological evidence of rejection was reported by Martinez et al (15). This could be due to the fact that they analysed intragraft IL-4 mRNA expression without reference to the associated liver function which we did. An alternative explanation might be that differences in immunosuppressive protocols have influenced the results, as we have not used anti-thymocyte globulin or FK506. It is evident that Th2 cytokines can antagonize synthesis as well as effector functions of Th1 cytokines, and visa versa, significantly affecting the result of the overall immune response (11-13). In the present study, IL-2 mRNA transcription was rarely observed in rejecting livers concomitant with IL-4 mRNA while, in contrast, in the spontaneously resolving rejections, a high incidence of IL-4 mRNA expression was seen in the absence of detectable IL-2 mRNA levels. In experimental transplantation, a state of tolerance has been characterized by several investigators as requiring the presence of Th2 cytokines, with relative dearth of Th1 cytokines in grafted tissue (9-11,33,34). Evidence for such a role of IL-4 in clinical kidney transplantation was recently reported by Kusaka et al (35). Peripheral blood cells from a chimeric renal transplant patient who discontinued all immunosuppressive drugs produced high amounts of IL-4 protein. These data suggest that circulating donor cells might control the specific immune response towards the transplanted organ by interfering into the cytokine cascade. After liver transplantation chimerism is a common phenomenon (36,37) and therefore this hypothesis might also be

relevant for our patients. However, it can not be excluded that other activation pathways or cytokine cascades are also involved in this phenomenon. In conclusion, our results show that the function of liver transplants remains unimpaired despite histological signs of rejection, provided IL-4 mRNA is expressed at the same time. This suggest that also in man, cells committed to Th2 function control cell mediated immune responses *in vivo*.

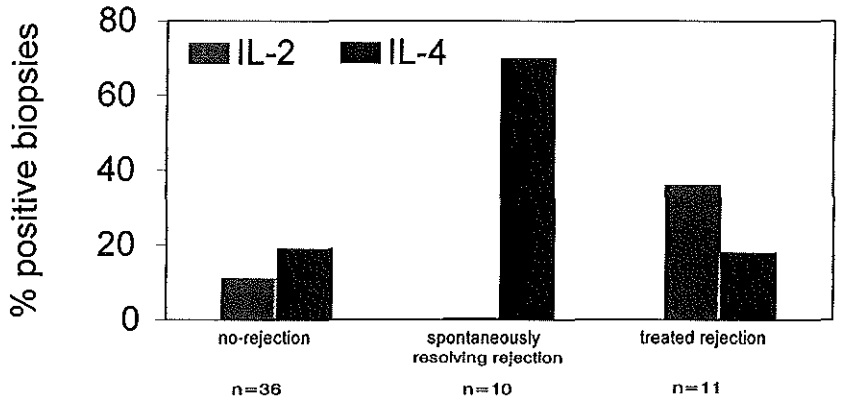

Figure 2. The biopsy without histological evidence of rejection as diagnosed during review taken from a patient with clinical evidence of rejection was omitted from the no-rejection group. Analysis was only done in biopsies both without histological and clinical evidence of rejection. IL-2 and IL-4 mRNA expression in liver allograft biopsies without histological and clinical evidence of rejection, in biopsies with spontaneously resolving rejections and in biopsies with cellular infiltrate in the subsequently treated patients. Data expressed as the percentage of liver biopsies containing mRNA for IL-2 or IL-4. The proportion of liver biopsies expressing IL-4 mRNA was significantly higher in the spontaneously resolving rejections compared to either treated rejections ($p=0.03$, Fisher's exact test) or to biopsies without evidence of histological or clinical rejections ($p<0.01$). The presence of IL-2 transcripts was characteristic for liver biopsies with rejection of subsequently treated patients

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Chapter 9

INCREASED INTRAGRAFT IL-15 mRNA EXPRESSION AFTER LIVER TRANSPLANTATION

This chapter is based on the article published in:

Clin Transplantation (in press)

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ABSTRACT

Acute rejection is a T-cell mediated process associated with intragraft IL-2 production. However, the observation that rejection also occurs in the absence of IL-2 implies that T-cell activation is not merely dependent on local IL-2 production. IL-15, a macrophage derived cytokine associated with T-cell activation could also be involved in liver allograft rejection. To study T-cell/macrophage interactions at the molecular level, we measured intragraft mRNA expression of the T-cell derived cytokine IL-2 and the macrophage derived chemokine IL-15 in post-transplant liver biopsies (n=33) and in non-transplanted control liver tissue (n=5) by competitive template RT-PCR. We analyzed biopsies without evidence of rejection (n=12), with spontaneously resolving histological rejection (n=10), or with histological rejection accompanied with clinical rejection (n=11) defined by rising serum bilirubin and aspartate amino transaminase levels. IL-15 mRNA expression was present in the majority of post-transplant liver biopsies (91%, 30/33) and was significantly upregulated as compared with non-transplanted liver tissue (p=0.005, Mann Whitney U test). However, the intragraft IL-15 mRNA levels did not differentiate between the 3 post-transplant biopsy groups. In contrast to intragraft IL-15 mRNA expression, IL-2 mRNA transcription was measured in the minority of the post-transplant liver biopsies (15%, 5/33), not detectable in control specimens and almost exclusively found in rejection biopsies concurrent with graft dysfunction (36%, 4/11 vs 1/22; p=0.03, Fisher exact test).

Our results show an increased intragraft IL-15 gene expression after liver transplantation. This phenomenon is independent of the clinical status of the allograft and indicates constant macrophage activation. When IL-15 is also produced during this process it may participate in T-cell dependent donor-directed immune responses, thereby explaining the occurrence of rejection in the absence of IL-2.

INTRODUCTION

A major manifestation of acute rejection is the mixed infiltrate of inflammatory cells in the transplanted liver. Phenotyping studies showed that infiltrates mainly consists of macrophages, T-cells, eosinophils and rarely B cells and NK cells (1,2). Both infiltrated

macrophages and T cells are capable of expressing a large number of cytokines important for their function as a mediator in the host defence: the non-immune and specific immune response. During rejection, mRNA expression of both macrophage- and T-cell derived cytokines are upregulated. Tumor necrosis factor-α, IL-1β, and IL-6 are mainly produced by macrophages, as a result of the non-specific inflammatory response. IL-2, interferon (IFN)-γ, and IL-5 secreted by T-cells reflecting the specific immune response (3-7). Despite the fact that liver allograft rejection is mediated by T-cells, these studies also showed that these rejection episodes are not always regulated by the classical T-cell activator IL-2. This observation suggests that other cytokines may induce T-cell proliferation and differentiation. Studies in experimental transplant settings showed that T-cell growth factors produced by non T-cells are also able to induce and mediate rejection (8,9).

IL-15, a recently described T-cell growth factor produced by macrophages and not by lymphocytes shares many biological activities with IL-2. Like IL-2, IL-15 stimulates the proliferation of natural killer cells, mitogen activated T-cells and cytotoxic T-cells, serves as a chemoattractant for T-cells, and has stimulatory activity for the induction of B-cell proliferation and differentiation (10-14). These remarkable similar biological activities can be explained by the fact that both IL-2 and IL-15 mediate their function through the common β and γ chains of the IL-2 receptor apart from their own unique α-chain (10). Several investigators have described a role for IL-15 in a variety of diseases associated with monocyte infiltrate such as rheumatoid arthritis (15), responses to infection (16), and acute rejection after kidney transplantation (17). Therefore, we postulate that IL-15 produced by liver infiltrated macrophages plays a significant role in T-cell recruitment and activation resulting in rejection. To test this hypothesis, we analysed intragraft IL-15 mRNA expression in previously IL-2 characterized biopsies that were taken at different phases of an immune response and in control non-transplanted liver tissues (6). Using a competitive template reverse transcriptase-PCR (RT-PCR) method, we measured the amount of intragraft IL-15 mRNA expression in biopsies without evidence of rejection, in biopsies obtained with histological evidence of rejection and not followed by clinical signs of rejection, and in biopsies with histological rejection that were accompanied with clinical rejection.

MATERIAL AND METHODS

Patients

We analysed IL-15 and ß-actin mRNA expression in 33 liver biopsy specimens (6-206 days post-transplant) obtained from 16 patients (6 male and 10 female). In these samples, IL-2 mRNA expression was determined as described recently (6). The primary indications for liver transplantation were acute liver failure, cholestatic liver disease, posthepatitic liver disease, post alcoholic liver disease, and cryptogenic liver disease. Biopsies were taken according protocol or on indication in case of deteriorating liver function (table 1). Immediately following procurement, a portion of each biopsy was snap-frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. The biopsy specimens were processed in standard fashion, stained with haematoxylin and eosin, periodic acid-Schiff, and iron.

All samples were obtained from patients who had received a HLA-mismatched cadaver donor liver. Standard immunosuppression consisted of azathioprine, cyclosporin A and low-dose methylprednisolone. As controls we used non-transplanted liver tissue obtained from 5 different individuals.

Table 1. Characteristics of the liver biopsies and biochemical liver function parameters

biopsy group	number of biopsies	days pTX[1] median (range)	bilirubin (μmol/l) median (range)	ASAT (U/l) median (range)
no-rejection	12	24 (6 - 206)	46 (8 - 288)	23 (11 - 101)
histological rejection spontaneously resolving	10	12 (7 - 26)	111 (32 - 310)	35 (14 - 113)
histological and clinical rejection	11	17 (6 - 215)	178 (35 - 318)	90 (14 - 230)

[1], days post-transplant (pTX)

Management of rejection

A clinical diagnosis of rejection was suspected and considered likely if: (1) there was an increase in bilirubin and/or transaminases in successive days; (2) there was a decrease in the amount and colour of bile; and (3) other events were excluded radiologically, microbiologically or virologically.

Cellular rejection was reported according to the Snover criteria (18). In brief, grade 0, lymphocytic or mixed portal infiltrate, < 50% damaged bile ducts, no endothelialitis; grade 1, as above, with endothelialitis; grade 2, lymphocytic or mixed portal infiltrate, > 50% damaged bile ducts, with or without endothelialitis; grade 3, acute rejection plus arteritis, paucity of bile ducts, or central hepatocellular ballooning with confluent dropout of hepatocytes. Treatment for acute cellular rejection was given if the clinical diagnosis was considered to be probably correct and if there was histological evidence of cellular rejection. Patients

received methylprednisolone for treatment of acute rejection at a dosage of 1000 mg/day for 3 consecutive days and r-ATG or OKT-3 monoclonal antibodies for steroid-resistant or severe rejection.

mRNA Isolation and cDNA reaction

For RT-PCR analysis, specimens were snap-frozen in liquid nitrogen and stored at -80°C. Messenger RNA extraction and transcription was performed as described previously (6). Briefly, total RNA was extracted from snap-frozen samples by a modification of the guanidinium method, as described by Chomczynski and Sacchi (19). Specimens were homogenized in 500 µl 4 mol/L guanidinium-isothiocyanate in the presence of 20 µg poly A (Boehringer, Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1], respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 mol/L sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000xg at 4°C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate treated-H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from the isolated RNA with 0.5 µg hexanucleotides (Promega Corporation, Madison, WI) and transcribed with 1000 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 100 µl. The reaction mixture consisted of 20 µl 5x MMLV-RT buffer (250 mmol/L Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 5 µl (10 mM) dNTP, 400 U of RNAsin (Promega) and 10 µl 0.1M DTT.

Polymerase Chain Reaction

Sequence specific primers were used for amplification of the human IL-15 gene (sense primer 5' CAA GTT ATT TCA CTT GAG TCC GGA G 3' and anti-sense primer 5' TTC TAA GAG TTC ATC TGA TCC AAG G 3'). Recently, we described the intragraft IL-2 mRNA analysis in the above mentioned post-transplant biopsies (6). PCR primers detecting transcripts for the human house-keeping gene ß-actin (Clontech Laboratories, Palo Alto, CA) were used as an internal control to monitor mRNA extraction and cDNA amplification. For qualitative analysis, 0.5 µl cDNA sample was added to 99.5 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ (IL-15) or 1.5 mM $MgCl_2$ (IL-2, ß-actin), 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence specific primers. Each reaction mixture was overlaid with 75 µl mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a DNA thermal cycler (Biomed-60, Germany) under the following conditions. For IL-15: after a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The last cycle was extended with 7 min at 72°C. For ß-actin: after a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, MI) for 4 h at 37°C. Messenger RNA

from this positive control was extracted as described above. Negative controls consisted of omission of reverse transcriptase from the cDNA synthesis reaction for each biopsy followed by amplification in PCR with the IL-15 and ß-actin primers, and the use of diethylpyrocarbonate treated-H_2O as no-template reaction. After amplification, 16 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, UK) by electroblotting, and hybridized with $\gamma^{32}P$ labelled specific probes which are located across the splice-site (ß-actin, Clontech LAbs; and IL-15 5'TGT CCT TCT GCA GAT TGA CAA TGC CCG TCT 3'). Hybridization was detected by autoradiography and indicated the presence of IL-15 and ß-actin mRNA expression in the original biopsy.

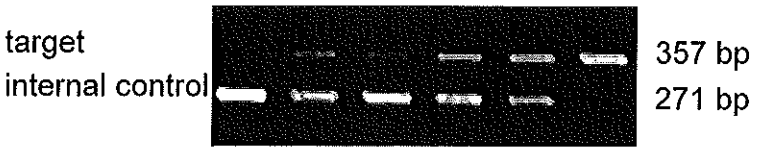
Competitive template RT-PCR

To estimate the relative initial amount of functional IL-15 mRNA in biopsies a competitive RT-PCR assay was used and comparison was made against the house-keeping ß-actin gene. The latter gene is assumed to be expressed at a constant level in biopsies. To obtain a standard curve for IL-15 and ß-actin, known amounts of internal control fragment were added in different dilutions to constant amounts of sample cDNA for competitive co-amplification with specific primers. The internal control was designed to generate a PCR product of a different size to allow differentiation between the amplified target and internal standard. Dilutions of the competitor template, ranging from 5 ag to 5 fg were co-amplified with constant amounts of sample cDNA. Following PCR, using conditions as described previously, the amplification products were analysed by gel electrophoresis and the amount of products by the internal control and targets are determined for each individual reaction (figure 1A). The relative ethidium bromide intensity on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, NY). The logarithm of the ratio target/internal control is graphed as a function of the logarithm of the internal molar amount of the standard and at ratio 1, the starting concentration of IL-15 and ß-actin cDNA prior to PCR is assumed to be equal to the known starting concentration the competing internal control (figure 1B). The relative concentration of intragraft IL-15 gene transcripts were divided by the relative concentration of ß-actin. This represents the amount of IL-15 mRNA transcripts corrected for the amount of mRNA used for reverse transcription and the efficacy of each reaction

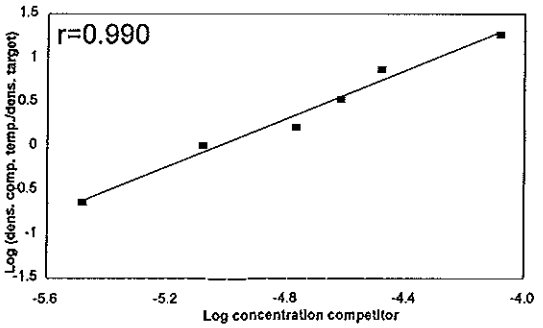
RESULTS

Qualitative RT-PCR analysis showed that IL-15 mRNA expression is present in all control specimens and in the majority of the post-transplant liver biopsies. In none of the control specimens (n=5), IL-2 mRNA was detectable. In the post-transplant liver specimens, IL-15 mRNA transcripts were present in 91% (30/33) and IL-2 mRNA in 15% (5/33) of the biopsies. We found no difference in the number of biopsies positive for IL-15 mRNA expression between biopsies without any signs of rejection, with

histological rejection not accompanied with clinical rejection, or with cellular infiltrate obtained from subsequently treated patients, 92% (11/12), 80% (8/10), 100% (11/11) respectively. However, for IL-2 a different intragraft mRNA profile was seen. IL-2 mRNA expression was predominantly found in biopsies derived from treated patients (36%, 4/11; p=0.03, Fisher's exact), not in biopsies with histological rejection only (0%, 0/10), and seldom in biopsies without any evidence of rejection (8%, 1/12).



1A



1B

Figure 1A. After electrophoresis, the ethidium bromide stained agarose gel shows two PCR products per lane representing discrete competitor (271 bp) and target (357 bp) for IL-15.

Figure 1B. The log of the ratio amplified IL-15 to competitor IL-15 is graphed as a function of the log of the known amount of competitor added to PCR reaction. At the point where target cDNA and internal control are in equivalence (i.e., ratio=1), the starting concentration of IL-15 cDNA prior to PCR is equal to the known starting concentration of the competing internal control.

We performed quantitative RT-PCR analysis in order to detect differences in IL-15 mRNA expression levels between the three post-transplant biopsy groups and the control specimens. Using the competitive template RT-PCR, we found no statistical difference among groups in expression of the positive control housekeeping gene β-actin. This indicates that the integrity of the mRNA for the three biopsy groups and the control specimens is the same (p=0.81, Krusal-Wallis). Relative amounts of initial IL-15 mRNA were individually normalized to the corresponding β-actin levels, which permitted more accurate comparison of IL-15 gene transcripts levels. The normalized IL-15 transcript levels were clearly different between liver biopsies and control specimens. The level of IL-15 mRNA was at least 10-fold greater in post-transplant biopsies than in non-transplanted control specimens (p=0.005, Mann Whitney U test, figure 2). However, the increased IL-15 transcript level was not associated with rejection. We measured comparable intragraft IL-15 mRNA levels between non-rejection biopsies versus biopsies with histological rejection (p=0.74, Mann Whitney U) or versus biopsies from patients with clinical rejection (p=0.88). Moreover, the IL-15 mRNA expression level was not elevated in the IL-2 negative rejections (figure 2).

DISCUSSION

In a previous study, we have shown that intragraft IL-2 and IL-4 mRNA expression could discriminate between different clinical circumstances after liver transplantation. Intragraft IL-2 mRNA expression was primarily found in biopsies associated with clinical rejection, while intragraft IL-4 mRNA expression was mostly measured in biopsies with spontaneously resolving rejections (6). In these biopsies we now have studied the contribution of intragraft IL-15 mRNA and compared the results with intragraft IL-15 mRNA expression measured in biopsies at time of immunological quiescence and in non-transplanted liver tissue.

Human IL-15, a cytokine with chemoattractant activity, is produced by monocytes/macrophages, other non T-cell types and measured at low levels in heart, lung, liver and kidney. IL-15 exerts overlapping biological activities with IL-2. An important feature of both IL-2 and IL-15 is the capacity to induce generation of cytotoxic

T-lymphocytes responsible for graft destruction (12). Intragraft IL-2 production is associated with T-cell mediated allograft rejection (20). However, the fact that rejection also occurs in the absence of IL-2 implies that T-cell activation is not only the result of local IL-2 production (21). IL-15, could therefore be an important factor by which T cells recruit to the graft and become activated T-cells.

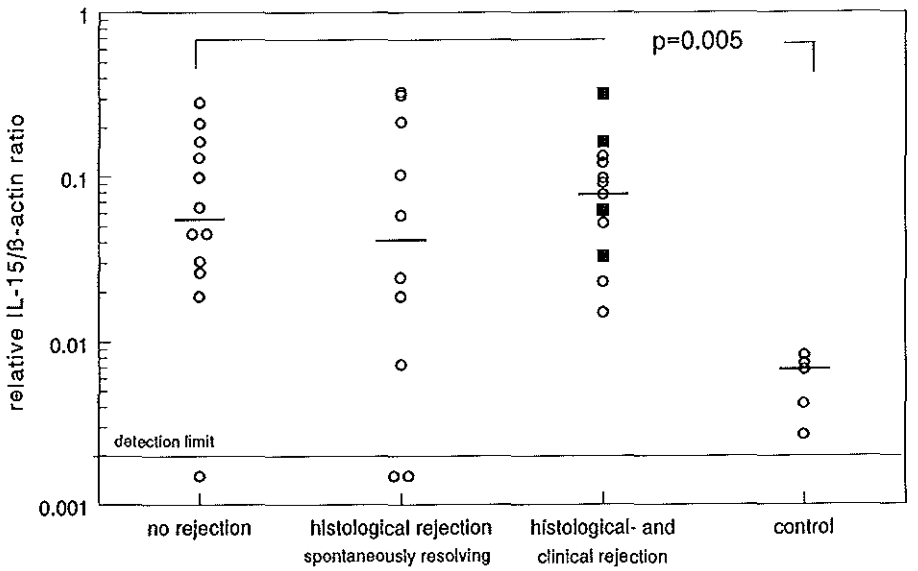


Figure 2. Intragraft IL-15 mRNA expression levels after clinical liver transplantation. IL-15 mRNA levels are depicted as a ratio of β-actin mRNA.

Quantitative analysis showed a significant increase of IL-15 mRNA expression in post-transplant specimens compared to non-transplanted control samples (p=0.005, Mann Whitney U test). Comparable intragraft IL-15 mRNA levels were measured in biopsies without rejection, in biopsies with histological rejection not followed by clinical signs of rejection, and in biopsies obtained from patients treated for rejection. Moreover, IL-15 transcript levels were not heightened in IL-2 negative rejecting specimens (o) compared to IL-2 positive rejecting specimens (■).

In the current study, we demonstrated that IL-15 mRNA is expressed in the majority of the post-transplant liver biopsies, irrespective of the rejection state. Nevertheless, intragraft IL-15 transcript levels were not indicative for rejection. However, post-

transplant IL-15 mRNA levels were significantly upregulated compared to non-transplanted liver tissue (p=0.005, figure 2). These differences could be related to the origin of IL-15 mRNA production. In non-transplanted liver tissue, Kupffer cells may contribute to IL-15 mRNA expression, whereas after transplantation, apart from these tissue specific macrophages, also graft infiltrated cells may transcribe IL-15 mRNA. IL-2 mRNA expression was almost exclusively measured in biopsies with cellular infiltrates obtained from patients that had to be treated for rejection. Moreover, we were unable to identify a relation between intragraft IL-2 mRNA expression and intragraft IL-15 mRNA levels. This observation suggests that IL-15 does not compensate for the lack of IL-2 during liver allograft rejection, since no increased intragraft IL-15 mRNA levels were measured. However, we must be cautious drawing this conclusion. Relatively "low" intragraft IL-15 mRNA amounts may be biological active. IL-15 production is predominantly regulated at the post-transcriptional level. Studies by Bamford et al. suggest that IL-15 mRNA may exist in translationally inactive pools (22). Cells may respond to stimuli by releasing the quiescent IL-15 message followed by translation and IL-15 protein production. Therefore, comparable intragraft IL-15 mRNA levels do not naturally lead to comparable bioactive IL-15 protein levels in the graft. In contrast to IL-2 mRNA transcription, the induction of IL-15 mRNA transcription can not be blocked by the immunosuppressive agent cyclosporin (23). Quantitative RT-PCR analysis in kidney biopsies showed that intragraft IL-15 transcript levels were increased in IL-2 negative clinical rejection episodes (17). Thus, indeed IL-15 may take over the role of IL-2 in clinical transplant settings. The discrepancy between the findings in liver and kidney transplantation can neither be explained by differences in the technique used nor on parameters of clinical rejection. Both studies used a competitive template RT-PCR method to determine intragraft IL-15 mRNA levels and in both instances clinical rejection was diagnosed by abnormal graft function which was confirmed by examination of a core needle biopsy. Variations in immunosuppressive strategies, organ specific immune responses or even a combination of these factors could be responsible for the observed differences. On the other hand, our data show that IL-15 may not be such an important cytokine in the rejection process after liver transplantation.

The process whereby inflammatory cells are recruited to inflammatory sites such as an transplanted organs can be the result of chemokine activities (24). Chemokines upregulate adhesion molecules present on leucocytes and promotes the migration of the adherent cells across the endothelium. Recently, Adams et al. reported that macrophage-derived chemokines are involved in the recruitment of T-cells into the transplanted liver (25). These chemokines, macrophage inflammatory protein-1α and macrophage inflammatory protein-1ß were already expressed within hours after transplantation. Histopathologic analysis in liver and kidney biopsies showed that inflammatory cells also infiltrate grafts with stable function (17,26). This ongoing level of inflammation might be responsible for the constant increased transcription of IL-15. Although it is impossible to distinguish Kupffer cells from graft infiltrating macrophages, it is likely that IL-15 is involved in inflammatory processes after transplantation such as recruitment and activation of T-cells. The importance of cytokine production by liver infiltrated macrophages was shown by Hoffmann et al (27). In this study, clinical rejection was characterized by a significant portal tract and central venous infiltration by TNF-α positive macrophages. Nevertheless, the mechanism whereby macrophage-derived cytokines interact with T cells in acute rejection processes remains obscure. It is likely that other - presently unknown - macrophage, derived cytokines contribute to liver allograft rejection.

In conclusion, contrary to IL-2, we found no indication for a significant role of IL-15 in the rejection process after clinical liver transplantation. The increased intragraft IL-15 mRNA level after transplantation suggests that cells producing IL-15 play a critical role in the ongoing inflammatory response, it may constantly modulate T-cell influx.

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Chapter 10

SUMMARY AND DISCUSSION

SAMENVATTING

SUMMARY

Transplantation biology is an exiting and rapidly developing field with the intention to understand immune mediated mechanisms responsible both for rejection and for the induction of tolerance. Initially, two facets of the response to an allograft were studied: the cellular and the antibody response. The cellular anti-donor response was investigated *in vitro* by proliferation- and cytotoxicity assays. These studies showed that T-lymphocytes are the key mediators of the donor-directed immune process. Recently, due to the enormous development in molecular biology, an immune response within the allograft can be studied *ex vivo*, thus, without *in vitro* manipulations. Moreover, this technology made it possible to study interactions between immune competent cells and transplanted tissue instead of just focussing on donor lymphoid cells which is characteristic for the *in vitro* assays. Interaction of the T-cell receptor with donor peptides, present in the groove of major histocompatibility complex molecules on the surface of antigen presenting cells, results in production of multiple transcriptional factors including cytokines, chemokines, growth factors and their receptors. These mediators typically function within an immunologic micro-environment after binding to their specific receptors on target cells. On basis of their cytokine production pattern T-cells can be divided into functionally different subsets (1). In experimental transplant immunology, type 1 cells are associated with cell mediated immune responses while type 2 cells may suppress the allogeneic immune response (2). So, the function of type 1 cytokines (IL-2, IFN-γ) is antagonistic to the activity of type 2 cells (IL-4, IL-10).

The studies presented in this thesis were initiated to elucidate those parts of the cytokine network that are involved in clinically significant issues: acute and chronic rejection, spontaneous down-regulation of an immune reponse, and the effectiveness of anti-rejection and prophylaxis treatment. In addition, we analyzed the kinetics of type 1 and type 2 mRNA and protein production by "primed" graft infiltrated T-lymphocytes in comparison to the time course of T-cell activation by "naive" peripheral blood mononuclear cells.

Chapter 1 summarizes the current literature on cytokine measurements by RT-PCR in clinical transplant settings. It became evident that neither rejection nor graft acceptance are restricted to stringent intragraft cytokine mRNA profiles. Lack of a clear association is probably caused by a number of factors, contributed by both the donor as well as the recipient. Moreover, these factors may have an immunological (e.g. HLA, blood transfusions) and/or non-immunological (e.g. age, ischemia damage) background which all have the potential to interfere in the T-cell activation cascade resulting in different intragraft cytokine mRNA profiles. Analysis by RT-PCR also showed that apart from T-cell derived cytokines, historically associated with graft rejection, cytokines secreted by other cell types are involved in the inflammatory response after transplantation. Chemokines such as MCP-1, MIP, RANTES, produced by macrophages and activated endothelial cells may initiate the T-cell dependent alloresponse.

In **chaper 2** the aim of the thesis is formulated.

Chapter 3 shows that the kinetics for IL-2 and IL-4 mRNA and protein production by "naive" peripheral blood mononuclear cells and by "primed" graft infiltrating lymphocytes are different. In comparison to peripheral blood mononuclear cells, donor-specific IL-2 and IL-4 mRNA and protein production by graft infiltrating lymphocytes was found immediately upon *in vitro* stimulation. After stimulation with donor antigens, detectable IL-2 protein levels secreted by graft infiltrating lymphocytes were already measured after 4 h versus 28 h by peripheral blood mononuclear cells. This implies that graft infiltrating lymphocytes are in a more "activated" state. In addition, these data suggest that "primed" T-cells home in the graft. During rejection, the activation grade of cytokine producing T-cell might vary between individual patients. This is based on the observation that each bulk culture of graft infiltrating cells had its own characteristic mRNA and protein profile. In these cultures, the donor-specific IL-2 mRNA signal was significantly higher than the IL-4 mRNA signal resulting in abundant donor-specific IL-2 protein levels ($p=0.002$).

The involvement of the above characterized cytokines (IL-2, IL-4) and of IL-6 and IL-10 in acute immune responses was studied in routinely taken endomyocardial biopsies (EMB) after heart transplantation. In **chapter 4** we demonstrate that, in

contrast to IL-10 mRNA expression, IL-2, IL-4 and IL-6 gene transcription are associated with immune responses after transplantation. A strong correlation between intragraft IL-2 mRNA expression and histologically proved rejection was found. In the majority of EMB taken during rejection IL-2 mRNA expression was present, whereas in EMB obtained during immunological quiescence IL-2 mRNA transcription was hardly detectable, 76% versus 4% respectively (p=0.001). Although, in this study, the presence of intragraft IL-4 and IL-6 mRNA expression did not discriminate between rejection and immunological quiescense, the results suggests that both IL-4 and IL-6 are involved rejection responses (IL-4, 62% versus 35%; and IL-6, 81% versus 39%, respectively). We conclude with regard to cardiac allograft rejection, that analysis of IL-2 gene transcription is a good candicate to monitor the acute immune response on the molecular level. The combination of histology and molecular biology will provide detailed information of the rejection process, not only morphological features of rejection are determined but also the activation state of the graft infiltrating cells.

Blockade of the IL-2 mRNA transcription by anti-IL-2R monoclonal antibodies may provide an opportunity to interrupt the T-cell activation cascade. The data presented in **chapter 5** show that induction therapy with a mouse anti-IL-2R monoclonal antibody (BT563), directed against the α-chain of the IL-2R complex, hardly influenced the allogeneic immune response. Nevertheless, immunologic phenotyping and RT-PCR analysis showed that during rejection in the presence of circulating BT563, the IL-2/IL-2R signaling pathway is almost completely blocked, demonstrating that cardiac allograft rejection can be mediated by other growth factors than IL-2. On the other hand, BT563 therapy did not affect the intragraft IL-15 mRNA expression, suggesting that IL-15 might have overtaken the function of IL-2 in the cardiac rejection process.

At our center, intravenous gifts of high doses of glucocorticoids like methylprednisolone (MP) are given as first-line therapy for acute cardiac rejection. However, approximately 40% of the patients fail to respond to MP anti-rejection therapy (3). In **chapter 6** we tried to identify patients who are either MP resistant or MP sensitive on basis of the intragraft cytokine profile, and studied the effect of MP

anti-rejection therapy on the cytokine transcriptional level. During the first acute rejection episode, before MP anti-rejection therapy was given, the individual cytokine patterns were not helpful in differentiating between rejection that proved to be MP resistant or MP sensitive. None of the analyzed cytokines (IL-2, IL-4, IL-1ß, IL-6, TNF-α) or the high affinity IL-2Receptor (IL-2R) were indicative for steroid resistance or steroid sensitivity. However, intragraft cytokine mRNA patterns clearly reflected the efficacy of steroid anti-rejection therapy. After successful anti-rejection therapy, the overall intragraft cytokine mRNA expression was down-regulated. Intragraft cytokine mRNA expression became hardly detectable (IL-2: 0%; IL-4: 17%; IL-6: 17%) while a 10-fold decrease in the amount of mRNA of the constitutively expressed cytokines or cytokine receptors (IL-1ß, TNF-α, IL-2R) was measured. In contrast, intragraft cytokine mRNA expression of ongoing rejections was not influenced by MP anti-rejection therapy (IL-2: 88%, p=0.005; IL-4: 62%, p=0.14; IL-6: 88%, p=0.03) and IL-1ß, TNF-α and IL-2R levels remained stable or even increased (p=0.03). This study shows that cytokine mRNA expression profiles in pretreatment EMB are not a good parameter to determine the subsequent efficacy of MP anti-rejection therapy. However, these intragraft cytokine mRNA profiles are an extremely useful tool to determine the efficacy MP anti-rejection therapy and probably also of other immunosuppressive agents that act at the cytokine transcription level.

The relation between intragraft cytokine production and the development chronic rejection in cardiac allograft recipients was studied in **chapter 7**.

At our center, chronic rejection or graft vascular disease (GVD) is diagnosed by visual evaluation of the coronary angiogram at one-year posttransplant (4). One-year after transplantation, mRNA expression of IL-2, IL-4 and IL-6, IL-10, TGF-ß and PDGF-α was not different between patients with GVD and their controls without GVD. In contrast, RT-PCR analysis showed that development of GVD is associated with intragraft IL-2 mRNA expression during the first acute rejection episode. IL-2 mRNA expression was present in 77% of the EMB from patients with GVD versus 33% of the patients without GVD a one-year (p=0.03). In the studied patient group also the non-immunologic factors older donor hearts and prolonged ischemia time

were found to be associated with GVD. Recipients with GVD were transplanted with older donor hearts (median: 32 y versus 23 y, p=0.02) and had longer ischemia time (median: 193 min versus 141 min, p=0.002). However, no relation was found between these non-immunologic initiators of GVD and IL-2 positive acute rejections. Thus, non-immunologic factors and allogeneic immune responses may independently trigger GVD.

Chapter 8 deals with the issue: do cytokines play a role in processes responsible for down-regulation of acute cellular immune responses? To investigate this postulation, we analyzed IL-2 and IL-4 mRNA expression in biopsies obtained at time of spontaneously resolving liver rejection. This phenomena is characterized by histological signs of rejection that are not associated with deranged liver function parameters. Therefore, additional immunosuppression is not necessary. RT-PCR analysis demonstrated that the presence of IL-4 mRNA strongly correlated with uncompromised graft function in spite of concomitant histological signs of rejection. Significantly more of these biopsies (70%) expressed IL-4 mRNA compared to biopsies without any evidence of rejection (19%, $p<0.01$) or to biopsies concurrent with graft dysfunction (18%, p=0.03). In contrast, IL-2 mRNA expression was never detectable in biopsies with spontaneously resolving rejection and only occasionally in biopsies without any evidence of rejection (11%) whereas a significant proportion of the biopsies of the treated patients expressed IL-2 mRNA (36%). These results suggest that cells committed to type 2 function control cell mediated immune responses.

From the data described in chapter 8, it is obvious that a significant number of liver allograft rejections develop in the absence of IL-2 mRNA expression. **Chapter 9** shows that also the level of intragraft IL-15 mRNA transcription, a macrophage derived chemokine associated with T-cell activation, is not associated with cellular rejection after liver transplantation. However, irrespective of the rejection state, the posttransplant IL-15 mRNA levels were significantly upregulated compared to non-transplanted liver tissue (p=0.005). These data imply that in the ongoing inflammatory response after transplantation, IL-15 may serve as a chemoattractant for T-cells.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The studies described in this thesis used the RT-PCR technique in an attempt to characterize acute and chronic immune responses by measuring intragraft cytokine mRNA profiles. Results of these studies were evaluated in relation to histology, graft function, and coronary angiogram. Specific intragraft mRNA profiles were found in clinically different complications. Especially the novel applications to predict the development of early stages of chronic rejection after heart transplantation (chapter 7) and to discriminate between rejection and spontaneously resolving cellular infiltrates after liver transplantation (chapter 8) at the cytokine transcriptional level makes the RT-PCR technique an unique tool to monitor anti-donor immune responses in more detail. However, the review of the current literature (chapter 1) demonstrated that specific intragraft cytokine mRNA profiles may vary between patients, institutes, and are affected by immunosuppressive agents. One of the limitations of the RT-PCR technique is the lack of information about the cells that actually transcribe the cytokines. The recently developed *in situ* RT-PCR technique may resolve this limitation. This technique combines the advantages of *in situ* hybridization with the sensitivity of the RT-PCR, which makes it possible to localize rare mRNA expression within a heterogeneous cell population.

The data described in chapter 6 suggest that intragraft cytokine mRNA profiles need to be studied at the earliest time peroid after transplantation. The inflammatory immune response caused by ischemia, perfusion and the surgical procedure may trigger the donor specific immune response via the cytokine cascade, thereby influencing graft function. Analysis of cytokine production shortly after transplantation may gain insight in the regulation of early allogeneic responses which in turn may initiate chronic rejection. Therefore, intragraft cytokine mRNA profiles have to be analyzed in relation to both morphological and clinical features of rejection. The findings reported by the group of Hutchinson suggested that even before transplantation cytokine analysis can be used to identify patients at risk for rejection. It was found that cytokine production is influenced by a genetic determinant (5). For instance, the G-to-A polymorphism at position -308 in the TNF-α promotor induces increased TNF-α secretion.

In summary, intragraft cytokine mRNA profiles by the RT-PCR technique will provide the clinician additional information on the immunological activation status within the transplanted organ.

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SAMENVATTING

Voor patiënten met een slecht functionerend hart of lever bestaat er vaak maar één behandeling, namelijk transplantatie. De eerste geslaagde klinische lever- en harttransplantaties werden respectievelijk in 1963 en 1967 uitgevoerd door respectievelijk Thomas Starzl en Christian Barnard (1,2). Na een hoopvol begin bleek het resultaat van orgaantransplantatie in de eerste jaren echter niet erg succesvol. Veel patiënten overleden aan de gevolgen van afstotingen en infecties. Een belangrijke doorbraak in de transplantatie geneeskunde was het gebruik van cyclosporine A als afstotingsremmend geneesmiddel beschreven door Roy Calne in 1978 (3). Verdere vooruitgang zoals de ontwikkeling van biopsieprocedures (4) en de introductie op grote schaal van anti-T cel geneesmiddelen (5) zorgden ervoor dat heden ten dage transplantatie als behandelingsvorm geaccepteerd is.

Transplantatie-biologie is een vakgebied dat zich bezig houdt met onderzoek naar de oorzaken van afstotingen en de mogelijkheden van acceptatie van organen en weefsels. In eerste instantie werden twee facetten van de anti-donor reactiviteit bestudeerd: de cellulaire en de antilichaam respons. De cellulaire reactie tegen donorantigenen werd bestudeerd door middel van *in vitro* proliferatie- en cytotoxiciteitstesten met behulp van uit het transplantaat gekweekte cellen. Deze studies lieten zien dat ontvanger T-cellen een prominente rol vervullen bij de immuunrespons tegen donorcellen. Door de ontwikkelingen in de moleculaire biologie kan men nu deze immuunreacties ook rechtstreeks in het transplantaat bestuderen. Bovendien maak deze technologie het mogelijk om de interactie tussen immuuncompetente cellen van de ontvanger met getransplanteerde donorweefsel te onderzoeken. Interactie van de T-cel receptor met peptiden aanwezig in de groeve van het "major histocompatibility complex" van antigeen presenterende cellen resulteert in de produktie van diverse transcriptie factoren zoals cytokinen, chemokinen, groeifactoren, interferonen en hun receptoren. Deze mediatoren functioneren tijdens immuunreactiviteit na binding aan hun specifieke receptoren op de doelwitcellen. Cytokinen veroorzaken veranderingen in het celgedrag. Zij dienen voor de communicatie tussen cellen: vanuit het perifere bloed worden cellen het transplantaat ingetrokken (chemokinen), doen dienst als groeifactor, zorgen voor

proliferatie en differentiatie van cellen en beïnvloeden de expressie van "Human Leucocyte Antigens" en andere oppervlaktestrukturen op cellen. Op basis van het cytokinen-produktiepatroon kunnen T-cellen worden onderverdeeld in functioneel verschillende subtypen. Vanuit experimentele transplantatie-immunologie werd het duidelijk dat type 1 cellen betrokken zijn bij cellulaire immuunreacties zoals acute afstotingen, terwijl type 2 cellen deze allogene responsen kunnen onderdrukken. Dus de functie van type 1 cytokinen (IL-2, IFN-γ) is tegengesteld aan de activiteit van type 2 cytokinen (IL-4, IL-10).

Hoofdstuk 1 bevat een samenvatting van de literatuur over cytokinenbepalingen met behulp van de reverse transcriptase-polymerase chain reaction (RT-PCR) techniek in transplantaatbiopten. Dit overzicht laat duidelijk zien dat zowel afstoting als natuurlijke acceptatie van een orgaan niet tot stand komt via afgebakende cytokinen-mRNA-patronen. Dit kan diverse oorzaken hebben, waarbij niet alleen ontvanger- maar ook donorfactoren een rol spelen. Bovendien kunnen deze factoren een immunologische (bv. HLA verschillen, bloedtransfusies) en een niet-immunologische (leeftijd, ischemie- en reperfusieschade) achtergrond hebben. Ook is duidelijk dat naast T-cel cytokinen ook mediatoren vanuit andere celtypen bepalend kunnen zij voor ontstekingsreacties na transplantatie. Voorbeelden zijn MCP-1, MIP, IL-6 en TNF-α, die door geactiveerde macrofagen en/of endotheelcellen geproduceerd kunnen worden en vervolgens afstotingsreacties kunnen uitlokken.

Hoofdstuk 2 beschrijft de vraagstellingen van de in dit proefschrift beschreven studies. Kort samengevat: het doel was het onderzoeken van die gedeeltes van het cytokinen-netwerk, die betrokken kunnen zijn bij klinisch relevante vraagstukken zoals acute en chronische afstoting, natuurlijke onderdrukking van immuunreacties, en preventie of behandeling van een afstoting. Bovendien hebben wij ons afgevraagd hoe de kinetiek van type 1 en type 2 transplantaat infiltrerende T-lymfocyten en perifere lymfocyten verliep op messenger RNA (mRNA) en eiwit-nivo. De analyse beschreven in **hoofdstuk 3** laat zien dat er verschil bestaat in kinetiek van zowel IL-2 en IL-4 mRNA als van eiwit-produktie door "naïve" perifeer

mononucleaire cellen vergeleken met die van "primed" transplantaat infiltraat infiltrerende lymfocyten. Kort na *in vitro* stimulatie met donorantigenen produceerden de transplantaat infiltrerende lymfocyten zowel IL-2 en IL-4 mRNA en eiwit. Perifere mononucleaire cellen waren daar niet toe instaat. Dit suggereert dat de transplantaat infiltrerende lymfocyten al geactiveerde cellen zijn. Verder kwam in deze studie naar voren dat, bij afstoting na harttransplantatie, iedere kweek van transplantaat infiltrerende lymfocyten zijn eigen karakteristieke donor-specifieke mRNA en eiwit-produktiepatroon heeft.

De studie beschreven in **hoofdstuk 4** laat zien dat immunologische processen na harttransplantatie gepaard gaan met verhoogde cytokine-mRNA-produktie met name van IL-2, IL-4 en IL-6 in het getransplanteerde orgaan. Bovendien bleek dat IL-2 mRNA expressie voornamelijk aanwezig was tijdens acute afstoting, 76% versus 4% in biopten zonder afstoting ($p=0.001$). Voor IL-4 en IL-6 mRNA expressie werd slechts een trend waargenomen, terwijl IL-10 mRNA expressie altijd in de biopten kon worden aangetoond. Op grond van deze bevindingen concluderen wij dan ook dat de bepaling van IL-2 mRNA expressie in harttransplantatiebiopten een belangrijke bijdrage kan leveren voor diagnostische doeleinden.

Het voorkomen van IL-2 mRNA transcriptie zou er toe kunnen leiden dat de T-cel activatie cascade wordt onderbroken. **Hoofdstuk 5** beschrijft dat blokkade van de IL-2Receptor (CD25) door het monoklonale antilichaam BT563 resulteert in verminderde aantallen vroege acute afstotingen na harttransplantatie. In biopten, afgenomen tijdens de behandeling, werden slechts sporadisch IL-2 mRNA en CD25 positieve cellen aangetoond. Omdat ondanks blokkade van de IL-2/IL-2R route toch afstotingen optraden, blijkt dat het afstotingsproces naast IL-2 ook door andere cytokinen kan worden gereguleerd. Aangezien BT563 behandeling geen effect had op de T-cel groeifactor IL-15, doet dit vermoeden dat IL-15 de functie van IL-2 in het afstotingsproces kan overnemen.

Het is duidelijk dat, ondanks verschillende, soms nog experimentele geneesmiddelen, harttransplantatie patiënten vaak meerdere perioden van acute afstoting doorstaan. In eerste instantie worden deze behandeld met hoge doses steroïden. Echter, bij veel patiënten (± 40%) faalt deze behandeling en blijft het

afstotingsproces doorgaan. **Hoofdstuk 6** beschrijft het effect van hoge doses steroïden op de cytokinen-mRNA-expressie in getransplanteerd hartweefsel. Vóór afstotingstherapie bleek het cytokinen-mRNA-expressiepatroon noch de mate van expressie te differentiëren tussen steroid resistente en steroid gevoelige behandelingen. Na afstotingstherapie werden wel significante verschillen gemeten tussen beide afstotingsvormen. In biopten met histologisch aantoonbare doorgaande afstoting werd nog steeds IL-2 mRNA expressie aangetoond en wel in 88% van de biopten, terwijl na succesvolle behandeling in geen van de biopten IL-2 mRNA werd gemeten (p=0.005). Ook IL-4 en IL-6 mRNA expressie was nauwelijks meer in deze biopten aantoonbaar (beide 17%), terwijl dit wel het geval was bij steroid resistente afstotingen waar IL-4 mRNA meetbaar was in 62% (p=0.14) en IL-6 in 88% (p=0.03). Bovendien werd in biopten van succesvol behandelde afstotingen de mate van mRNA expressie van IL-1ß, TNF-α, en IL-2R significant lager (p=0.03). Deze studie laat zien dat het cytokinen-mRNA-expressiepatroon en nivo weliswaar geen voorspellende waarde heeft voor de werkzaamheid van steroïden als afstotingstherapie, maar dat het succes van de behandeling hier wel aan af te lezen is.

Op de langere termijn is chronische afstoting één van de belangrijkste complicaties na transplantatie. Na harttransplantatie manifesteert chronische afstoting zich als versnelde coronairsclerose. Al na één jaar zijn in 26% van de coronairen afwijkingen aantoonbaar. In **hoofdstuk 7** wordt de bijdrage van immunologische en niet-immunologische factoren beschreven op het ontstaan van deze versnelde coronairsclerose. Analyse van immunologische factoren liet zien dat op het moment van de diagnose versnelde coronairsclerose, één jaar na transplantatie, het cytokinen-mRNA-expressiepatroon in getransplanteerd weefsel niet differentieert tussen patiënten met en zonder deze vaatafwijkingen. Daarentegen gaf het cytokinen-mRNA- expressiepatroon tijdens de eerste acute afstoting kort na transplantatie wel een aanwijzing voor de ontwikkeling van versnelde coronairsclerose. MessengerRNA expressie van IL-2, maar niet dat van de andere cytokinen, was geassocieerd met chronische afstoting op één jaar. Bij patiënten met versnelde coronairsclerose werd het IL-2 gen aangetoond in 77% van de biopten

tegen 26% van de samples afkomstig van patiënten zonder deze vaatafwijkingen (p=0.02). Ook niet-immunologische risicofactoren zoals koude ischemietijd (mediaan 193 versus 191 min; p=0.002) en donorleeftijd (mediaan 32 versus 23 jaar; p=0.02) bleken geassocieerd met het ontstaan van versnelde coronairsclerose. Tussen de immunologische en niet-immunologische factoren werd echter geen relatie aangetoond. De in hoofdstuk 7 beschreven resultaten laten dan ook zien dat ischemie, donorleeftijd en IL-2 positieve acute afstotingen risicofactoren zijn die onafhankelijk van elkaar versnelde coronairsclerose kunnen induceren.

"Kunnen cytokinen immuunprocessen ook onderdrukken" is de vraagstelling van de studie, beschreven in **hoofdstuk 8**. Om deze vraag te beantwoorden hebben wij een klinisch model onderzocht waarbij immunologische reacties niet automatisch leidde tot transplantaatfunctieverlies. Kort na levertransplantatie kunnen identieke histologische beelden passend bij acute afstoting zowel gepaard gaan met tekenen van levercelverval als met verbeterende biochemische leverfunctieparameters. In de bij deze histologische beelden behorende biopten hebben wij de cytokinen-mRNA-expressie geanalyseerd. Wij vonden significante verschillen in biopten met histologische afstotingen afkomstig van transplantaten met en zonder biochemische verschijnselen van afstoting. IL-4 mRNA expressie werd in 70% van de biopten met spontaan verbeterende parameters aangetoond en slechts in 18% van de biopten met een vergelijkbare histologie afkomstig van transplantaten met verslechterde leverfunctieparameters. Bovendien werd in deze laatste transplantaten vaker IL-2 mRNA gemeten: 36% versus 0% in biopten met infiltraat zonder klinische afstoting. Het antwoord op de vraag: "kunnen cytokinen de acute immuunrespons ook onderdrukken" is dus JA. De resultaten van deze studie suggereren dat type 2 cellen in de getransplanteerde lever in staat zijn tot het onderdrukken van de type 1 afhankelijke afstotingsreacties.

Een ander opvallend resultaat van de studie beschreven in hoofdstuk 8 is, dat afstoting van levertransplantaten vaak plaatsvindt in afwezigheid van IL-2 mRNA expressie. Andere T-cel groeifactoren zouden hierbij dus een rol kunnen spelen. **Hoofdstuk 9** beschrijft de analyse van IL-15 mRNA expressie in pre- en post-transplantatie leverweefsel. In post-transplantatie biopten was het IL-15 mRNA

expressie nivo significant hoger dan in pre-transplantatie leverweefsel (p=0.005). Hoewel geen relatie tussen de mate van IL-15 mRNA expressie en afstoting gevonden werd, zou alleen al de aanwezigheid van de T-cel groeifactor IL-15 (in afwezigheid van IL-2 en IL-4 [zie hoofdstuk 8]) afstoting tot gevolg kunnen hebben.

De studies, beschreven in dit proefschrift, laten zien dat met behulp van reverse transcriptase-polymerase chain reaction (RT-PCR) technieken het mogelijk is om interacties tussen de transplantaat infiltrerende cellen en het donorweefsel op mRNA nivo te bestuderen. Processen als acute en chronische afstoting, spontane onderdrukking van een immuunrespons en werkzaamheid van immunosuppressiva kunnen worden vervolgd aan de hand van karakteristieke cytokine-mRNA-expressiepatronen in het transplantaat.

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DANKWOORD

Dit proefschrift is het resultaat van samenwerking tussen de afdelingen Interne I (Transplantatie en Nefrologie), het MBDI laboratorium van de afdeling Virologie, Cardiologie, Thoraxchirurgie, Interne II, Pathologie en Heelkunde. Mijn dank gaat dan ook uit naar velen.

Allereerst wil ik mijn promotor Willem Weimar bedanken voor zijn ideeën, enthousiasme en motiverende opmerkingen. Jouw liefde voor onderzoek leidde ertoe dat naast de cytotoxiciteit van de transplantaat-infiltrerende-cellen ook de cytokinen-mRNA-expressie diende te worden bestudeerd. De grote vrijheid die jij mij bij dit onderzoek gaf, heb ik zeer gewaardeerd. Ik had nooit verwacht dat "GIPCA" metingen, mijn eerste aanraking met cytokinen onderzoek, de opstap voor een proefschrift zouden worden.

Mijn leermeester op het PCR terrein en co-promotor Bert Niesters dank ik voor de plezierige samenwerking tussen jouw MBDI-lab en het Transplantatie-lab. Het openstellen van het MBDI-lab voor cytokinen-mRNA onderzoek heeft naast dit proefschrift ervoor gezorgd dat cytokinen-mRNA metingen een belangrijk onderdeel van de research zijn geworden binnen de afdeling transplantatie/nefrologie.

De collega's van de interne I, lab en kliniek: dank ik voor de prettige manier waarop onderzoek bedreven wordt binnen onze afdeling. Mijn soms botte opmerkingen hebben jullie er gelukkig niet van weerhouden om enthousiast te blijven. Wendy, Cécile en Lisette zonder jullie cytokinen-mRNA metingen zou het proefschrift een stuk dunner zijn geworden. De resultaten van al deze bepalingen waren dan ook van hoge kwaliteit en hebben tot vele presentaties op congressen geleid: bedankt. Chris: bedankt dat ik van jou de immunohistochemie heb mogen leren. Ik hoop dat wij samen de *in-situ* cytokinen metingen van de grond krijgen. Len: alhoewel jij van de functionele testen bent, volgde jij het cytokinen onderzoek op de voet. Ik hoop dat onze plezierige samenwerking ertoe mag leiden dat het laboratorium verder zal groeien. Volgens mij is de interactie tussen functionele- en cytokinen testen een unieke combinatie. Mijn slaapmaatje tijdens congressen: Nicole, bedankt voor je bijdrage bij het kinetiek onderzoek. Teun: zonder jouw hulp bij de diverse projecten was het mij nooit gelukt om de cytokinen-mRNA resultaten te correleren aan

klinische gegevens. Nicolet: bedankt voor het corrigeren van mijn abstracts en manuscripten. Willem, Len, Nicolet, Cees, Willy, René, Alice, Lisette, Chris, Nicole, Franciska, Nancy, Wendy, Teun, Iza, Cécile, Paula, Barbara, Ronella, Frans, Marij en Hester, allemaal bedankt voor de goede sfeer op het lab, tijdens borrels, etentjes, en labweekends.

Veel dank gaat ook uit naar alle medewerkers van het MBDI-lab, zonder iemand te kort te willen doen, dank ik in het bijzonder Edwin Fries en Jolanda Voermans. Edwin: jij leerde mij de PCR technieken waarmee de resultaten beschreven in dit proefschrift zijn behaald. Daarnaast was het jou en Jolanda nooit te veel om bij te springen als ik weer eens langs kwam waaien.

"Wat hebben mijn patiënten aan al die mooie testen van het lab" is een karakteristieke opmerking van Aggie Balk. Deze kritische kanttekening heeft ertoe geleid dat er sinds kort een samenwerkingsproject tussen de afdelingen cardiologie en de interne I van start is gegaan. Bedankt dat jij de lab-crew ook de andere kant van transplantatie onderzoek wilt laten zien.

De samenwerking met de medewerkers van de afdeling pathologie tijdens de BT563-studie heb ik zeer gewaardeerd. Met name Pieter Zondervan en Frieda van der Ham bedank ik voor hun hulp bij het opzetten van de immunohistochemie.

Mijn beide paranimfen, Chris en Willy, bedankt voor jullie hulp om van de 20st mei een prachtige dag te maken.

Lieve Ineke, bedankt voor de vele kopjes koffie, als ik weer eens achter de computer zat, maar vooral voor je steun en vertrouwen dat het proefschrift er uiteindelijk zou komen.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 4 juni 1962 te Gouda. Na het behalen van het MAVO diploma aan de Koningin Wilhelmina MAVO te Waddinxveen, werd in 1978 begonnen met de HBO-A opleiding tot klinisch chemisch analist aan het van 't Hoff Instituut te Rotterdam. Na het afronden van deze opleiding in 1981 was zij tot eind 1984 werkzaam op de afdeling Experimentele Chirurgie van de Erasmus Universiteit te Rotterdam. Van 1985 tot eind 1986 werkte zij mee aan het Ataxia Telangiectasia onderzoek van de afdeling Genetica. Hieropvolgend was zij gedurende een jaar in dienst van de Daniel den Hoed Kliniek op de afdeling Immunologie. Vanaf eind 1987 tot op heden, is zij werkzaam, aanvankelijk als analist, later als hoofdanalist, op het Transplantatie Laboratorium van de afdeling Inwendige Geneeskunde I van het Academisch Ziekenhuis Rotterdam-Dijkzigt. In 1992 begon zij met haar promotie-onderzoek naar de rol van cytokinen bij immunologische processen na klinische orgaantransplantatie onder leiding van Prof. Dr W. Weimar. Dit onderzoek vond in nauwe samenwerking plaats met Dr H.G.M. Niesters van het Moleculair Biologisch Diagnostisch Laboratorium van de afdeling Virologie. In 1995, tijdens het promotie-onderzoek behaalde zij het HLO diploma klinische chemie aan de Hogeschool Rotterdam en Omstreken. De resultaten van het cytokine mRNA onderzoek in getransplanteerd weefsel staan in dit proefschrift beschreven.