The role of anti Interleukin-2Receptor monoclonal antibody (BT563) in the prevention of acute rejection after organ transplantation.

Gelder, Teunis van

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The role of anti Interleukin-2 Receptor monoclonal antibody (BT563) in the prevention of acute rejection after organ transplantation.

De rol van monoclonale antilichamen gericht tegen de Interleukine-2 Receptor (BT563) ter voorkoming van acute afstoting na orgaantransplantatie.

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CONTENTS

List of abbreviations

Chapter 1 Anti Interleukin-2 Receptor monoclonal antibodies in organ transplantation.
Introduction

Chapter 2 In vitro effects of BT563, an anti Interleukin-2 Receptor monoclonal antibody.
Transplant International 1994;7(Suppl 1):S556-S558

Chapter 3 In vivo effects and pharmacodynamics of prophylactic antirejection therapy with an anti Interleukin-2 Receptor monoclonal antibody (BT563) after heart and kidney transplantation.
Transplantation Proceedings 1995;27:856-868
Immunopharmacol 1995;30:237-246

Chapter 4 A randomized trial comparing safety and efficacy of OKT3 and a monoclonal anti Interleukin-2 Receptor antibody (BT563) in the prevention of acute rejection after heart transplantation.
Transplantation (in press)

Chapter 5 Intragraft monitoring of rejection following prophylactic treatment with monoclonal anti Interleukin-2 Receptor antibody (BT563) in heart transplant recipients.
Journal of Heart and Lung Transplantation 1995;14:346-350

Chapter 6 A double-blind, placebo-controlled study of monoclonal anti Interleukin-2 Receptor antibody (BT563) administration to prevent acute rejction after kidney transplantation.
Transplantation 1995;60:248-252

Chapter 7 Reversal of heart allograft rejection with monoclonal anti Interleukin-2 Receptor antibody.
Lancet 1992;339:873 (Letter)

Chapter 8 Summary and conclusions

Chapter 9 Samenvatting en conclusies

Dankwoord

Curriculum vitae

List of abbreviations:

AP	Alkaline Phosphatase
ATN	acute tubular necrosis
CD	Cluster of Differentiation
BSA	bovine serum albumin
CIT	Cold Ischemia Time
CMP	cardiomyopathy
CMV	cytomegalovirus
CsA	cyclosporin-A
EBV	Epstein-Barr Virus
EMB	endomyocardial biopsy
FITC	fluorescein isothiocyanate
GAM	Goat anti Mouse
GVHD	Graft-versus-Host-Disease
HAMA	human anti-mouse antibodies
HTX	heart transplantation
Ig	Immunoglobuline
IL-2R	Interleukin-2Receptor
ISHLT	International Society for Heart and Lung Transplantation
kDa	kiloDalton
MoAb	Monoclonal Antibody
MLR	Mixed Lymphocyte Reaction
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	phycoerythrin
PRA	Panel Reactive Antibodies
r-ATG	rabbit-AntiThymocyteGlobuline
RIA	radioimmunoassay
RTX	renal transplantation
SEM	standard error of the mean
sIL-2R	soluble Interleukin-2Receptor
TBS	Tris Buffered Saline
TCR	T-cell receptor

Chapter 1

Anti-IL2 Receptor monoclonal antibodies in organ transplantation

This chapter is a modified version of a review on this subject, published as "Anti-IL2Receptor monoclonal antibodies in organ transplantation." in Developments in Nephrology - Leiden; Boerhaave course 1995.

1.0 Introduction

Results of solid organ transplantation have improved steadily over the last decades. However, both acute and chronic rejections are still frequently found. Detailed single center studies have shown that the presence or absence of acute rejection episodes is one of the most powerful predictors of chronic rejection and late graft failure[1 2 3]. In order to reduce the incidence of acute rejection in the immediate postoperative period and to promote long-term hyporesponsiveness to donor allo-antigens, the use of induction therapy with anti T-lymphocyte antibodies has been widely promoted[4]. Especially OKT3, a murine monoclonal anti-CD3 antibody, has been used for this reason in some regimens with the additional advantage of delaying the requirement for cyclosporin, thereby reducing the risk of nephrotoxicity[5 6]. The use of OKT3, however, has several limitations[7]: first dose reactions may be life-threatening[8] (cytokine release syndrome[9 10]), the procoagulant-effect may lead to early graft loss[11], and infections[12 13] and neoplasms[14] may complicate the clinical course as a result of excessive immunosuppression. Polyclonal anti T-lymphocyte antibody preparations additionally have the problem of batch-to-batch variability and the risk of serum sickness[15].

To avoid the drawbacks of aselective immunosuppression of anti-pan T-cell preparations, monoclonal antibodies against specific T-cell activation determinants have been developed[16]. Using monoclonal antibodies T-cell responsiveness might be manipulated in an attempt to achieve donor-specific tolerance[17]. Antibodies directed against specific molecules that are only expressed after antigenic stimulation and involved in the early steps toward clonal expansion of alloreactive precursors will only interfere with the resulting antigen-committed cells. An example are the anti Interleukin-2Receptor monoclonal antibodies[18].

1.1 Interleukin-2 and the Interleukin-2Receptor

In the seventies it was established that T-cells are the source of a substance with mitogenic activity, sustaining long term T-cell growth[19]. The term "T-cell growth factor"[20] was changed into interleukin-2, according to the international

lymphokine nomenclatura, when the molecular properties of IL-2 were defined[21 22] and cDNA clones were isolated and sequenced[23].
IL-2 acts on several cells of the immune system, such as B lymphocytes, macrophages and natural killer cells[24]. It has a pivotal role in the antigen-specific clonal proliferation of T-lymphocytes, where it stimulates growth in an autocrine and paracrine fashion via interaction with IL-2Receptors[25]. These IL-2Rs have all the characteristics of true cytokine receptor molecules, including high affinity, saturability, ligand specificity and target cell specificity. The IL-2R originally was thought to consist of an α(p55)- and ß(p75)-chain[26 27]. Recently an additional molecule was found, the γ(p64)-chain[28], associated with the ß-chain, and neccessary for the formation of functional intermediate and high affinity IL-2Rs[29 30]. A mutant T cell line that has lost expression of the gamma chain but retains the alfa and beta chains has been shown to have also lost the ability to respond to IL-2[31].
Although the low-affinity IL-2Rα chain is able to bind IL-2, this proces is not functionally followed by signal transduction[32]. In contrast, the intermediate- and high-affinity IL-2R both can internalize IL-2. The large cytoplasmic region of the IL-2Rß chain, consisting of 286 amino acids, is the most likely candidate involved in driving the IL-2 dependent signal pathways[33].
The in vitro proliferative response of resting lymphocytes to IL-2 can be divided into two phases[34]: in the first phase concentrations of IL-2 high enough to bind intermediate affinity receptors are required. Cells respond by expressing IL-2Rα de novo and by increasing IL-2Rß chain expression. After 16 hours significant numbers of high-affinity receptors are expressed and subsequent proliferation can be maintained by low-dose IL-2 (sufficient to bind high affinity receptors but not enough to appreciably bind intermediate affinity receptors). Upon antigen or mitogen stimulation the α chain expression is highly upregulated (30-100 fold), whereas the ß chain induction is only 3-4 fold increased[35]. Three days after polyclonal lymphocyte stimulation maximal IL-2R levels are measured. Thereafter the level of IL-2R declines progressively, proliferation eventually ceases while most cells return in the resting phase of the cell cycle (G_0 or early G_1) by 12 to 14 days after initiation of the culture[36]. This experiment shows that IL-2R expression is transient. In vivo studies using polymerase chain reaction to examine IL-2 gene transcription within fine needle aspiration biopsies from renal

allograft recipients with an acute rejection,taken on a daily basis, also showed transient IL-2 gene expression[37]. More support for an important role of IL-2 in allograft rejection was obtained from studies where IL-2R positive cells were found to be present in rejecting grafts[38 39 40], and from a study that showed strong binding of radio-labeled IL-2 to high-affinity IL-2Receptors in acute rejection[41].

1.2 Anti IL-2R mAb in animal models.

In 1982 a murine IgG_{2a} monoclonal antibody, termed anti-Tac, was found to bind to the IL-2R α chain and to inhibit IL-2 induced T-cell proliferation[42]. Blockade of the α or the ß chain blocked the high-affinity IL-2R and inhibited internalization of the ligand IL-2[43]. Because high-affinity IL-2R are expressed solely by activated T-cells and not by resting T-cells, antibodies against IL-2R have been used to achieve a selective immunosuppression in allograft recipients. As these anti IL-2R monoclonals do not lyse or activate target cells no toxic syndrome is likely to occur.

In 1985 Kirkman et al[44] were the first to demonstrate increased heart and skin allograft survival in anti IL-2R mAb (M7/20)[45] treated mice, thereby clearly emphasizing the role of the IL-2R in the mechanism of graft rejection. In addition to preventing rejection they were also able to reverse ongoing rejection with M7/20.

Likewise, the murine anti IL-2R mAb ART18 was found to prolong allograft survival and abrogate acute rejection in a rat cardiac allograft model[46 47]. Using IL-2R targeted therapy in the rejecting host, donor-specific T-suppressor cells were thought to be spared, possibly because this subset of T-cells would not express IL-2R during early rejection[48]. Transfer of spleen cells from ART-18 treated hosts into untreated syngeneic animals prolonged graft survival, supposedly as a result of these T-suppressor cells, that inhibit the effector function of host cells[49]. Such donor-specific suppressor cells were also found in studies with the anti IL-2R mAb Tü69[50]. Anti IL-2R mAbs have further been tested in pancreatic islet[51], kidney[52] and neural[53] allograft transplantation in several rodent models, repetitively showing prolonged allograft survival in the treated animals. Some authors claim to find a synergistic effect in combining anti IL-2R monoclonals and cyclosporin[54]. This is explained by the fact that

cyclosporin acts at a proximal phase of the immune activation, whereas anti IL-2R monoclonals target antigen activated cells already generated[55].
Not all mAbs directed against the IL-2R are equally effective. A comparison of the functional potential of six mAbs directed against the mouse IL-2R p55 unit resulted in identification of several epitope clusters[56]. The differences in epitope-recognition may have important clinical consequences[57,58].

1.3 Anti IL-2R mAb in humans.

Shortly after the publication of the results of studies with anti-IL-2R mAb in animal models, clinical studies in humans were published. The group from Nantes, France, used 33B3.1, a rat IgG2a mAb to prevent rejection after kidney transplantation. In a prospective trial 100 consecutive recipients of primary cadaveric renal allografts were randomized for prophylactic treatment with 33B3.1 or ATG (Anti Thymocyte Globuline) for the first 2 weeks after transplantation[59]. Cyclosporin was started at the end of these 2 weeks. The clinical tolerance of 33B3.1 appeared to be good, in contrast to ATG administration, which in 32% of the cases had to be interrupted for reasons of clinical intolerance. During the 14 day treatment period 6 rejections occurred in the 33B3.1 group, compared to only 1 in the ATG-group. The rejection-incidence at 3 months was nearly identical in both groups (31% vs 26% in the ATG-group), whereas infections were more frequent in the ATG-group. Similar results were obtained with 33B3.1 as prophylactic treatment after a second kidney transplantation[60]. The same group published a third study[61], in combined kidney-pancreas transplantation, where also a higher rejection-incidence was found in the 33B3.1-group compared to a control group of ATG-treated patients.
Kirkman et al.[62] (Boston, USA) performed an open-labelled randomized trial with anti-Tac in primary renal allograft recipients. Transplanted patients received either anti-Tac for the first 10 days together with low dose cyclosporine, azathioprine and prednisone, or triple drug treatment only. The anti-Tac treated group had a significantly longer time to the first rejection-episode, but the overall number of rejection-episodes was not significantly reduced. During the prophylactic treatment period in the anti-Tac group 5 rejections occurred. Infectious complications were evenly distributed. During the first ten days, the

percentage of peripheral blood T cells expressing IL-2R was not different between the anti-Tac treated and the control group[63]. This was in contrast to the Nantes study in which the IL-2R positive cells remained almost undetectable during administration of 33B3.1.
In a randomized study from Paris[64] using Lo-Tact-1 (rat IgG_{2b} anti-p55) after kidney transplantation, CD25-positive (=IL-2R bearing) blood cells also remained undetectable during the 14-day treatment period. Rejection incidences in the Lo-Tact-1 group and in the control group receiving ALG-induction treatment were similar, but infectious complications were less frequent in the anti IL-2R treated patients. On the basis of these results a large prospective randomized study using this monoclonal has been started in Paris.

In liver transplantation a randomized trial using Lo-Tact-1 compared to an OKT3-treated and to a control group receiving only triple drug therapy was done in Brussels, Belgium[65]. Disappointingly, the overall rejection rates during the first 3 months in the 3 groups were similar, although the percentages of steroid-resistant rejection episodes proved to be lower in the 2 groups that were treated with monoclonals. Because of a higher CMV-incidence in the OKT3 treated group it was concluded that the combination of the triple drug treatment with Lo-Tact-1 had a better risk-benefit ratio in induction immunosuppression after liver transplantation.
Another prophylactic study of the mAb YTH-906 in 100 liver transplant recipients in Cambridge and London, UK, did not result in differences in rejection, infection or survival either[66]. The Heidelberg-group (Germany) presented data of a pilot study using BT563 (a murine IgG_1 anti IL-2R mAb) in liver transplantation, where a spectacular decrease in rejection incidence was found[67]. Because of the small numbers and a historical control group their conclusion must however be regarded with caution. Nevertheless, using the same antibody, BT563, the liver transplant group in Berlin also achieved a lower incidence of early rejections compared to a historical control group treated with ATG[68 69 70], a result that was confirmed in a randomized trial performed thereafter[71].

Limited experience was obtained in the use of anti IL-2R mAb for the treatment

of ongoing acute rejection. Cantarovich published results on 33B3.1 treatment for acute rejection in renal allograft recipients[72]. Only 2/10 patients responded immediately and unambiguously to 33B3.1, and 4/10 of the cases responded with delay. Suggested explanations for this high failure-rate are the possibility that there was too much IL-2 or insufficient mAb at the rejection site. It could also be that the host immune response leading to graft destruction is no longer under IL-2 control at the time of treatment, but under the control of alternative activation pathways. In contrast, anti IL-2R mAb treatment was successful in the treatment of acute steroid-resistant graft-versus-host disease using B-B10 (=BT563)[73 74 75] or humanized anti-Tac[76], although others using the antibody 2A3, had less positive results[77]. Carl[78] published results on successful reversal of steroid-resistant acute interstitial renal rejection using BT563, but this study suffered from severe methodological errors and can not be regarded as support for efficacy of anti IL-2R monoclonals in the treatment of acute rejection.
All of these murine or rat anti IL-2R monoclonals are immunogenic and elicit human anti-source antibodies in treated patients[79 80]. Some authors have reported reduced antibody formation against the mouse monoclonal OKT3 using concurrent administration of azathioprine[81] or cyclosporine[82] during OKT3 treatment. Another way to circumvent this problem is the use of chimeric and humanized antibodies, that are markedly less immunogenic and therefore have longer half lifes in vivo. A study with humanized anti-Tac in cynomolgus monkeys showed prolonged cardiac allograft survival in the animals treated with the humanized antibody compared to those treated with the murine monoclonal[83]. The efficacy and safety of these anti IL-2R monoclonals in human organ transplantation remains to be proven, especially in view of the long half life of humanized monoclonals that may lead to prolonged lymphocytopenia and over-immunosuppression.

The aim of this thesis is to investigate safety and efficacy of an anti IL-2R monoclonal (BT563) in the prevention of acute rejection after clinical heart and kidney transplantation. After having confirmed its potency in in vitro comparison with other anti IL-2R monoclonals, using inhibition of mixed lymphocyte reactions as a parameter, two large prospective clinical trials were started. In

kidney allograft recipients BT563 will be compared with placebo in a double-blind randomized trial. In heart transplantation BT563 will be compared with our current policy of OKT3 as a rejection-prophylaxis in a randomized, non-blinded trial.

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Chapter 2

In vitro effects of BT563, an anti Interleukin-2Receptor monoclonal antibody.

This chapter is based on an article published in Transplant International 1994;7(Suppl 1):S556-S558), entitled: In vitro and in vivo effects of BT563, an anti Interleukin-2Receptor monoclonal antibody, by Gelder van T, Daane CR, Vaessen LMB, Hesse CJ, Mochtar B, Balk AHMM, Weimar W.

2.1 Abstract

BT563, a murine anti IL-2R monoclonal antibody, was found to be more potent than anti-Tac in inhibiting proliferation in the mixed lymphocyte reaction. Results obtained with 33B3.1 in these experiments were similar to those with BT563. The anti IL-2R monoclonal antibodies 2A3, anti-Tac and 33B3.1 bind to an epitope of the same cluster on the IL-2R as BT563.

2.2 Introduction

Clinical organ transplantation has become a routine medical procedure. Although immunosuppressive therapy has improved considerably, graft rejection and complications of immunosuppression, such as the occurrence of infections and the induction of malignancies, still bother doctor and patient all too frequently. Monoclonal antibodies (MoAbs) directed against the Interleukin-2 receptor (IL-2R) are one of the results of the continuing search for better immunosuppressive agents[1]. The anti IL-2R MoAbs anti-Tac[2] and 33B3.1[3] are the two best documented examples of this new class of immunosuppressive agents. As a result of differences in the recognized binding site on the IL-2R the efficacy of these monoclonals may vary considerably[4]. Even within the p55 chain of the IL-2R several epitope clusters have been identified[5]. We describe in vitro results with the anti IL-2R MoAb BT563.

2.3 Patients and methods

BT563 (Biotest Laboratories, Dreieich - Germany) is a murine anti IL-2R MoAb of the IgG_1-kappa isotype[6]. Anti-Tac is a murine anti IL-2R IgG_{2a} MoAb[7] and 2A3 is another murine anti IL-2R IgG_1 MoAb (Becton Dickinson). 33B3.1 is a rat IgG_{2a} MoAb[3] (Immunotech, France). All four monoclonal antibodies recognize epitopes on the p55 chain of the IL-2R.

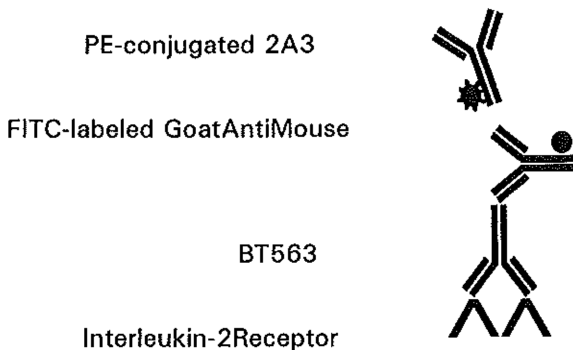
In vitro experiments.

Peripheral blood lymphocytes (PBL) of healthy blood donors were cultured in a mixed lymphocyte reaction (MLR) for 7 days in RPMI-1640 (Dutch modification) supplemented with 10% human serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 °C. Responder cells (5×10^4) were stimulated by 5×10^4 irradiated PBL (30 Gy) in triplicate in 96-well U-shaped

microtitre plates. MoAbs (BT563, 33B3.1 and anti-Tac) were added on day 0 to 6 after the start of the MLR. Proliferation was measured after 8 hours of ^{3}H-thymidine (1μCi/well) incubation. The effect of the MoAbs was determined as the percentage of inhibition of proliferation in the MLR.

2.4 Results

To monitor the effect of BT563 on peripheral blood we looked for an anti IL-2R MoAb that recognizes another epitope on the IL-2R. In the flow cytometric analyses we initially used unconjugated BT563 with fluorescein (FITC)-labelled goat anti-mouse serum (GAM-FITC) as a second step, and phycoerythrin (PE)-conjugated 2A3. With this technique 2A3-PE appeared to be bound to free binding sites of the goat anti-mouse (GAM) antibodies in the BT563 GAM-FITC complex, making it unsuitable for monitoring the effect of BT563. By direct labelling of BT563 or 2A3 with FITC this problem was solved.



Although initial experiments showed that after binding of FITC-conjugated BT563 it was still possible for 2A3 to bind the IL-2R, we could not reproduce these results in later experiments. This was most likely caused by too low BT563 concentrations in the first experiments, leaving free binding sites for 2A3. Subsequential binding studies with combinations of the four monoclonal antibodies (BT563, 2A3, anti-Tac, 33B3.1) showed that they all interfere with each others binding to the IL-2R, indicating that they recognize epitopes from the same cluster (Figure 2.1). Peripheral blood monitoring following BT563 treatment can therefore be performed using 2A3. Binding of 2A3 corresponds with free binding sites on the IL-2R.

Increasing concentrations of BT563 were first tested for their ability to inhibit proliferation in the MLR. As shown in Figure 2.2 lymphocyte proliferation was inhibited in a dose-dependent manner with maximal (86%) inhibition at concentrations above 500 ng/ml. The inhibition obtained with anti-Tac, also shown in Figure 2.2, was considerably less, with a maximal inhibition of 42.5%. Results with 33B3.1 were similar to those with BT563.

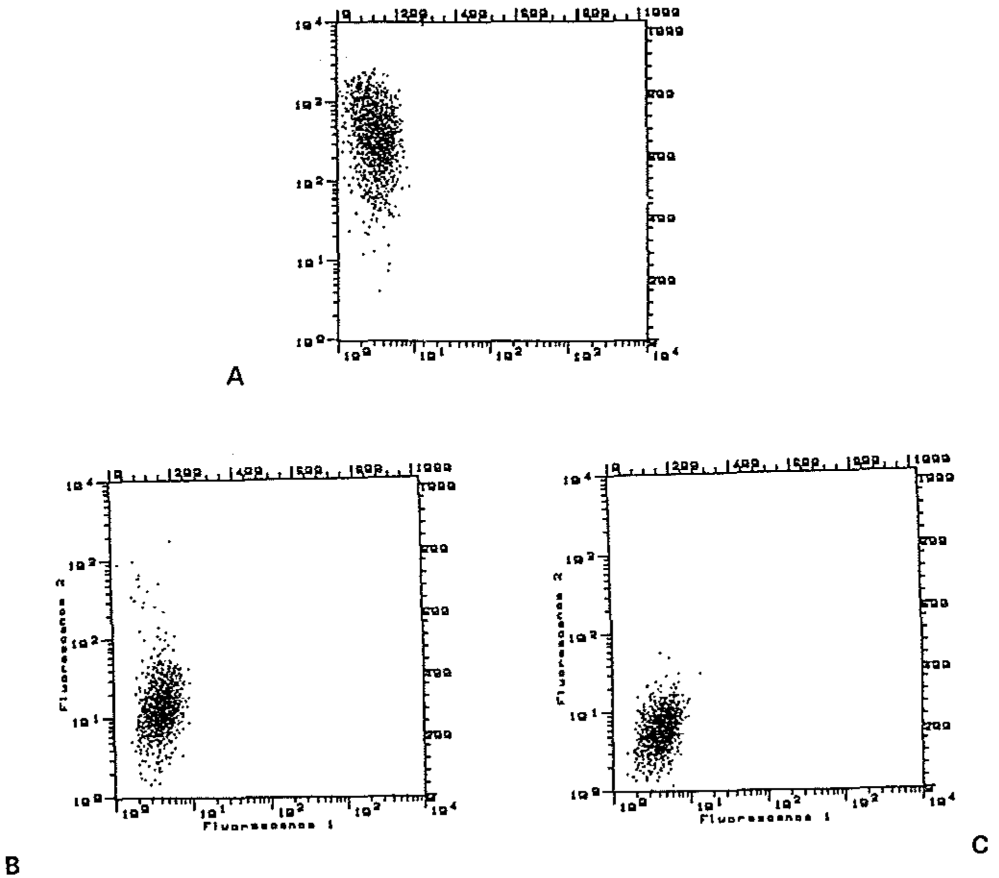


Figure 2.1ABC Flow cytometry images of binding of the monoclonal antibody 2A3 (y-axis) after BT563 administration (x-axis), using 20 ng/ml (A), 200 ng/ml (B) and 2000 ng/ml (C) BT563.

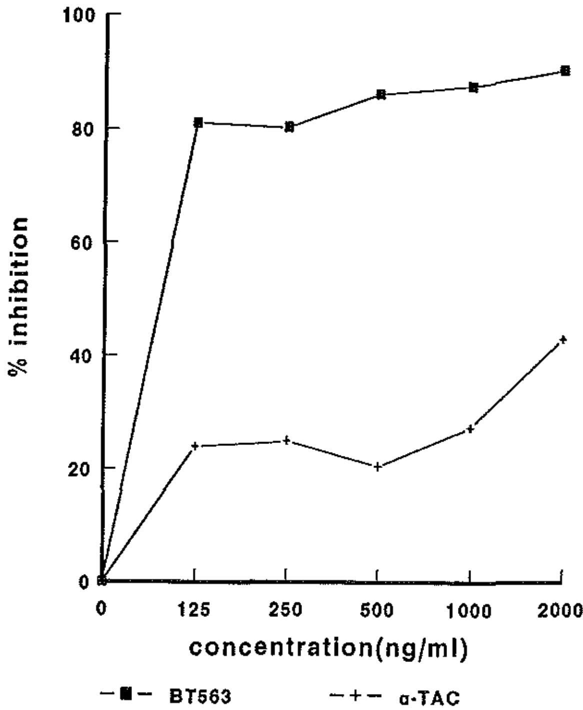


Figure 2.2 Inhibition of lymphocyte proliferation in the MLR by increasing concentrations of BT563 (■) and anti-Tac (+).

If the monoclonal was added to the MLR on day 1, 2, 3 or even 4, BT563 gave a maximal inhibition. Later addition no longer inhibited proliferation to this extent (Figure 3). Unlike BT563, addition of anti-Tac on day 4 did not result in inhibition to the same extent as on day 0 (decrease from 42.5% to 20%). Again, with 33B3.1 we found an inhibitory effect similar to that of BT563.

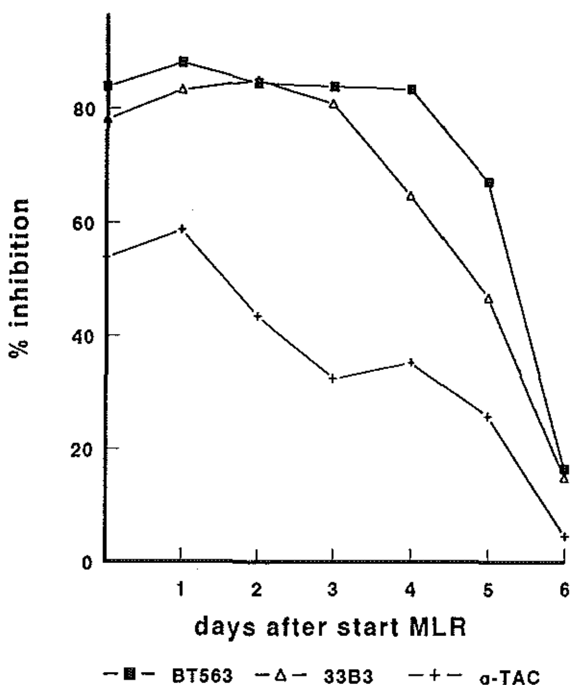


Figure 2.3 Inhibition of lymphocyte proliferation in the MLR by using three different monoclonal antibodies in a concentration of 2000 ng/ml (BT563 (■), 33B3.1 (▲), anti-Tac (+)), in relation to the day of addition after start of the MLR.

2.5 Discussion

BT563 is a murine IgG_1 anti IL-2R monoclonal antibody directed against the p55 subunit of the IL-2R. This monoclonal is able to induce an 86% inhibition of MLR proliferation and was shown to be more potent than anti-Tac (42.5% inhibition). Even if administered after 4 days of culture, BT563 was able to give a similar degree of inhibition as on immediate addition to the culture medium. In contrast, the administration of anti-Tac after 4 days resulted in only 50% of the initial inhibitory effect. Both experiments using 33B3.1 gave results similar to those with BT563.

These results suggest BT563 to be an effective inhibitor of IL-2R mediated lymphocyte activation. The clinical value of this new immunosuppressive drug is currently under investigation.

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Chapter 3

In vivo effects and pharmacodynamics of prophylactic antirejection therapy with an anti Interleukin-2Receptor monoclonal antibody (BT563) after heart and kidney transplantation.

This chapter is based on the following articles:

Peripheral blood monitoring during and after rejection-prophylaxis with a monoclonal anti Interleukin-2Receptor antibody in kidney and heart transplant recipients, by Gelder van T, Knoop CJ, Hesse CJ, Vaessen LMB, Balk AHMM, Yzermans JNM, Weimar W, in Transplantation Proceedings 1995;27:856-868.

and

Pharmacodynamics of prophylactic antirejection therapy with an anti-Interleukin-2Receptor monoclonal antibody (BT563) after heart and kidney transplantation, by Hesse CJ, Gelder van T, Vaessen LMB, Knoop CJ, Balk AHMM, Yzermans JNM, Jutte NHPM, Weimar W, in Immunopharmacology 1995;30:237-246.

3.1 Summary

A mouse monoclonal antibody (BT563) directed against the α-chain of the IL-2 receptor was administrated immediately after transplantation in a dose of 10 mg/day prophylactically to 30 heart transplant recipients and 42 renal transplant recipients to induce immunosuppression. Plasma levels increased to a plateau level of 5300 ng/ml in the heart and 5900 ng/ml in kidney recipients. BT563 plasma disappearance curves give a mean $T_{½}$ of 39 hour (range 14 - 112) and 42 hour (range 8 -122) for heart and kidney respectively. The CD25 marker (IL-2R) on peripheral blood lymphocytes was coated with BT563 within hours after the first gift and returned to normal within 0 - 20 days after the last gift. In heart transplant recipients more often $CD25^{+}$ cells were found in the presence of BT563 and more rejections occurred shortly after discontinuation of BT563 compared to the kidney recipients. Rejectors and nonrejectors within the heart transplant group did not differ with respect to the period that CD25 positive cells in peripheral blood were BT563 coated. In 56% of the patients a substantial IgM antibody response was detected. This response was similar for heart and kidney and not related to rejection. The frequency of IgG responses was low in both heart (13%) and kidney (21%) patients and the IgG response was not related with graft rejection or with antirejection treatment. Peripheral monitoring showed that monoclonal antibody plasma levels, antimurine antibody responses and coating of CD25 positive cells were not related with the clinical results. BT563 proved to be safe with respect to the generation of antimurine antibodies and, when given in combination with cyclosporin, is a therapy with a potential for high efficacy.

3.2 Introduction

The use of murine monoclonal antibodies (mAbs) in rejection prophylaxis or therapy is helpful in suppressing the recipient immune response during the first weeks after clinical transplantation as has been described extensively[1 2 3 4]. The most frequently used mAb is OKT3 (anti-CD3), a pan-T cell mAb with proven efficacy[5]. One of the drawbacks of the use of this mAb is the so-called cytokine release syndrome, associated with the first gift(s), resulting in fever, chills and hypotension in most patients. For that reason and the fact that all T-

cells irrespective of their involvement in rejection are affected, there is an ongoing search for more specific therapeutic strategies with less side-effects.
A more selective approach toward immunosuppression would be to target only activated T-cell clones via the interleukin-2 receptor. Interleukin-2 and the interleukin-2 receptor complex (IL-2R, CD25) play an important role in the activation and proliferation pathway of T-lymphocytes. The IL-2R complex consists of at least three distinct membrane components: the 55-kDa α-chain (IL-2Rα) or Tac antigen, the 70/75 kDa ß-chain (IL-2Rß) and a 64 kDa γ-chain (IL-2Rγ). Activated T-cells express the inducible IL-2Rα chain whereas resting cells and their precursors do not. The interaction of alloantigens with the T-cell antigen receptor complex (TCR) induces the expression of interleukin-2 and its homologous receptor on the T-cell surface resulting in proliferation of these cells. Disturbing the latter process by blocking the receptor with a monoclonal antibody should prevent the onset of the rejection process. Soulillou et al[6] and Kirkman et al[7] reported positive effects of prophylactic use of anti-IL-2Rα monoclonal antibodies on graft survival in renal transplantation. In the present study we report the in vivo consequences of prophylactic use of the mAb BT563 such as effectiveness of CD25 coating, mAb levels and half-life in plasma and human antimouse antibody (HAMA) responses. Blood samples were obtained from kidney transplant recipients in a BT563 versus placebo double-blind controlled study and from heart transplant recipients in a BT563 versus OKT3 study. Clinical results of both studies were reported in detail elsewhere[8 9].

3.3 Materials and methods.

Monoclonal antibodies. BT563 (Biotest Pharma, Dreieich, Germany), previously known as B-B10, is a murine mAb of the IgG1-kappa isotype and directed against the p55 or α-chain of the IL-2R complex[10] (Wijdenes et al. 1989). Orthoclone OKT3 (Cilag, Schaffhausen, Switzerland) is a murine IgG2a mAb directed against the T-cell receptor associated CD3-complex.

Patients. BT563 was given as rejection prophylaxis to 72 allograft recipients (30 heart and 42 kidney) in a daily dose of 10 mg in a sequential treatment scheme including cyclosporin A (CsA) and prednisone. Kidney transplant recipients received 10 daily doses of BT563 (days 0-9) and simultaneously CsA was

started at day 0. Initially, twelve patients participated in a pilot study with BT563. The other 30 kidney allograft recipients were treated with BT563 during a double-blind, placebo-controlled trial, of which the results are shown in chapter 6. In the kidney transplant recipients, cyclosporin was started on day 0 at a dose of 2 mg/kg per day i.v. until day 3, and at 8 mg/kg per day orally thereafter, aiming at whole blood levels of 300 to 350 ng/ml (radioimmunoassay). The diagnosis of acute rejection was made on clinical grounds by the responsible nephrologist, and was confirmed by core needle biopsy. Clinical indicators of acute rejection included rising creatinine, oliguria, fever and graft tenderness.

BT563 (10 mg/day) or OKT3 (5 mg/day) was given for 7 days (days 0-6) to the heart transplant recipients and CsA was given from the third postoperative day, in a dosage of 8 to 10 mg/kg per day, aiming at whole blood levels of 400 ng/ml. In addition heart transplant recipients received 50 mg azathioprine daily during the first week. Endomyocardial biopsies were performed weekly during the first 6 weeks after transplantation. These biopsy specimens were graded histologically according to the criteria of the International Society for Heart and Lung Transplantation[11]. Specimens graded as 3A, 3B or 4 were regarded to represent rejection episodes necessitating antirejection therapy. Acute rejections were initially treated with 1 gram methylprednisolone (Solu-Medrol) intravenously on three consecutive days. Steroid-resistant rejections were treated with a fourteen days course of rabbit anti-thymocyte globulin.

Flowcytometric analysis. Peripheral blood of the patients receiving BT563 or OKT3 was monitored daily for lymphocyte subsets during the first ten postoperative days and twice weekly thereafter until the uncoated IL-2R (CD25) or the TCR*α*ß-chains (WT31) respectively returned on the surface of the T-cells. T-cells were monitored with mAb against CD3, CD4, CD5, CD8 and HLA-DR (Immunotech, Marseille, France), TCR-*α*ß (WT31) and CD25 (2A3) (Becton-Dickinson, Mountain View, Ca, USA) conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The mAb 2A3 recognizes an epitope on the IL-2R*α* chain of the same cluster as BT563 and will only bind if BT563 coating has disappeared[12]. T-cell subsets were quantified by two colour analysis in a combination with anti-CD3 PE conjugated mAb (clone UCHT1) after BT563 or with an anti-CD5 PE conjugated mAb (IOT1a) after OKT3. Analyses were

performed on a FACScan flow cytometer (Becton-Dickinson) using Simulset software (Becton-Dickinson) with CD45 and CD14 as lymphocyte gate and as isotype controls murine IgG1 and IgG2a. In case of nonspecific binding of the mAbs, plasma was removed before staining by washing the blood cells with RPMI1640 + 1% bovine serum albumin (BSA). Persistent nonspecific staining was reduced by washing with the RPMI1640 solution containing an irrelevant mouse mAb.

Plasma BT563 and OKT3 levels were determined with an ELISA sandwich method. 96-wells enzyme immunoassay microtiterplates (Maxisorb, Nunc, Roskilde, Denmark) were coated overnight with 100 μl goat antimouse-IgG (Southern Biotechnology Associates, Birmingham, AL, USA) as capture antibody in phosphate buffered saline (5μg/ml). After washing with Tris buffered saline containing 0.05% Tween 20 (TBS/Tw), free binding sites on the plates were blocked with TBS/Tw containing 1% bovine serum albumin. A standard curve was constructed with parental grade BT563 and OKT3 in TBS/Tw/ containing 0.2 % BSA and ranged from 0.05 to 75 ng/ml. Appropriate plasma dilutions were selected to measure BT563 and OKT3 in the range of 20 - 10.000 ng/ml and 4 - 2.000 ng/ml respectively. Alkaline-phosphatase-conjugated goat antimouse-IgG antibodies (Southern Biotechnology Associates) were used as a detector antibody and p-nitrophenyl phosphate as substrate.

Human antimouse antibodies (HAMA's) were determined with an ELISA method using BT563 and OKT3 coated microtiterplates. The microtiterplates were coated overnight with 100 μl BT563 or OKT3 (5 μg/ml) in phosphate buffered saline. After washing and blocking with TBS/Tw, plates were filled with serial dilutions of plasma samples in TBS/Tw/0.2% BSA (dilutions 1:50 - 1:6400). The IgG and IgM HAMA's were quantified with Alkaline-phosphatase-conjugated goat antihuman IgG (Fc specific) or Alkaline-phosphatase-conjugated goat antihuman IgM (Fc5μ specific) (Jackson Immuno Research Laboratories, West Grove, PA, USA) and compared with a standardized plasma sample obtained from a patient with a clear IgG and IgM antibody response after BT563 treatment. IgM titre for this standard sample was set on 1000 arbitrary units (AU). The reference IgG anti-BT563 antibody concentration was measured as previously done for anti-OKT3 antibodies[13] and was assessed to be 154 mg/L . All samples were tested on BT563 (IgG1-isotype) and OKT3 (IgG2a-isotype)

coated microtiterplates to discriminate for isotype specificity.

Soluble IL2 receptor. Soluble IL2 receptor concentration in plasma was measured with an enzyme immunoassay (sIL-2r, Immunotech) according to the manufacturers instructions. The assay is a sandwich ELISA test using murine mAbs as coating and detecting antibodies.

Statistical methods. The significance of the differences between sample means was assessed by the Mann-Whitney U-test. Fisher's exact test was used to test associations between categorical variables.

3.4 Results.

Clinical results.

None of the 12 renal transplant patients who received BT563 in the pilot study did have an acute rejection. Of the 30 renal transplant recipients who received BT563 in the placebo-controlled trial only 3 patients experienced an acute rejection episode within the first three months. In all three $CD25^+$ cells had reappeared in the peripheral blood. At the end of the BT563 administration period in the heart transplant recipients 14/30 patients had an acute rejection (first week endomyocardial biopsy graded as 3A or 3B). This group of fourteen patients is further named rejectors and the other sixteen patients are further named non-rejectors. Rejections were treated with 1 gram methylprednisolone (Solu-Medrol) intravenously on three consecutive days. Steroid-resistant rejections were treated with rabbit anti-thymocyte globulin (RIVM, Bilthoven, The Netherlands).

BT563 plasma levels.

Comparable pharmacokinetics of BT563 were found in heart and kidney transplant recipients. The maximal plasma levels (5900 ± 200 ng/ml, mean of day 6 - 10, ± SEM) observed in the kidney transplant recipients were slightly higher than the mean peak levels (day 6 - 8) of the heart transplant patients (5300 ± 200 ng/ml) (Fig 1). The half-life values ($T_{½}$) of BT563 in blood calculated from the descending part of the disappearance curves of both groups of patients using a one-compartment model, were almost similar for kidney ($T_{½}$ = 42 ± 5 hr) and heart transplant patients ($T_{½}$ = 39 ± 3 hr). The half-life values of the individual patients were highly variable and ranged from 14 to 112

hours (median = 40 hr) in heart transplant recipients and from 8 to 122 hours (median = 48 hr) in kidney recipients and were not significantly different between both groups of patients (p = 0.1406). The differences in the plasma $T_{½}$ between the fourteen heart transplant rejectors (median 38.2 hr) and the sixteen non-rejectors (median 44.2 hr) were not quite significant (p=0.0559).

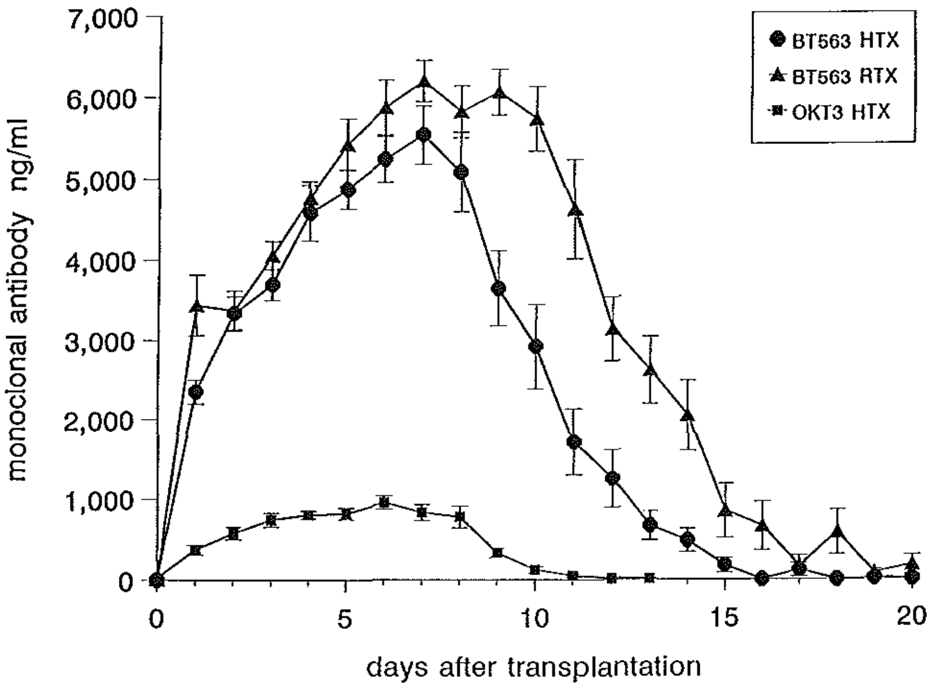

Figure 3.1 Pharmacokinetics of BT563 and OKT3 in heart transplant recipients (HTX) receiving BT563 (7 daily doses of 10 mg, n=30) or OKT3 (7 daily doses of 5 mg, n=20) and renal transplant recipients (RTX) receiving BT563 (10 daily doses of 10 mg, n=40). Mean trough levels ± SEM.

The effect of BT563 on peripheral blood cells.

The selective disappearance of free IL-2 binding sites by BT563 administration in heart transplant recipients is demonstrated in figure 3.2. The initial decrease of the total number of lymphocytes is an effect of steroid administration immediately after transplantation. The CD25^{+} cells are coated with the antibody within hours after the first gift of BT563 and coating persisted (last negative count for 2A3 binding) for a period of 13 days (median, range 8 - 19 days) in heart transplant recipients (N = 30). Blood samples were scored as positive if 10% or more of all T-cells had a positive staining for the CD25 marker. The median period of coating of CD25-positive cells in the group of rejectors was not significantly different from the group of non-rejectors (median 13.0 vs 13.5 days, p = 0.4410).

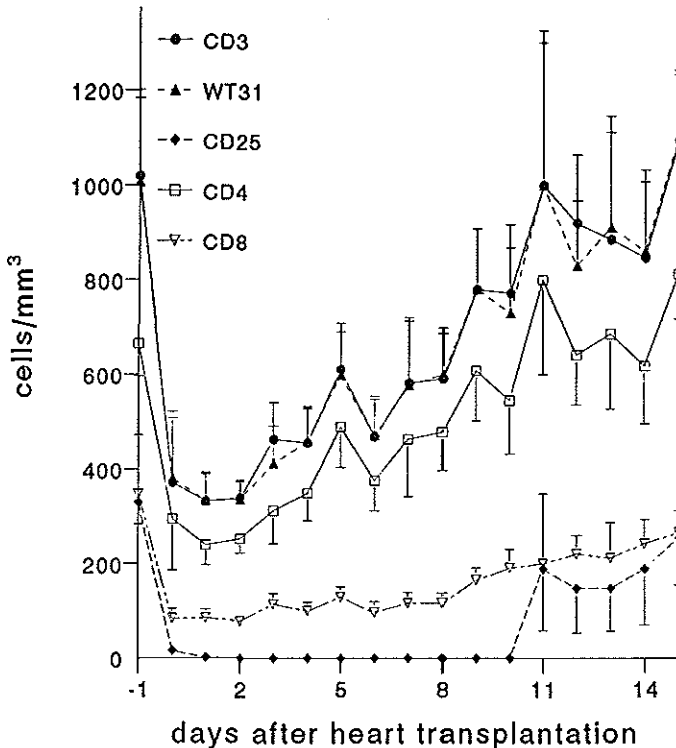

Figure 3.2 Monitoring the effect of BT563 on CD25 positive cells after heart transplantation. Mean concentrations and SEM of T-cells and T-cell subsets in the peripheral blood (n = 25).

In the kidney transplant recipients (N = 37) the median period of coating of CD25$^+$ cells in peripheral blood was 16 days (range 10 - 30 days).

After BT563 treatment in all patients CD25$^+$ cells returned to normal numbers (10% to 25% of the lymphocytes) without a significant increase in the number of T-cells (CD3$^+$ and WT31$^+$) indicating that the IL-2R indeed had been coated by the antibody.

Figure 3.3 shows the percentage of patients without free CD25$^+$binding sites at different days post transplantation.

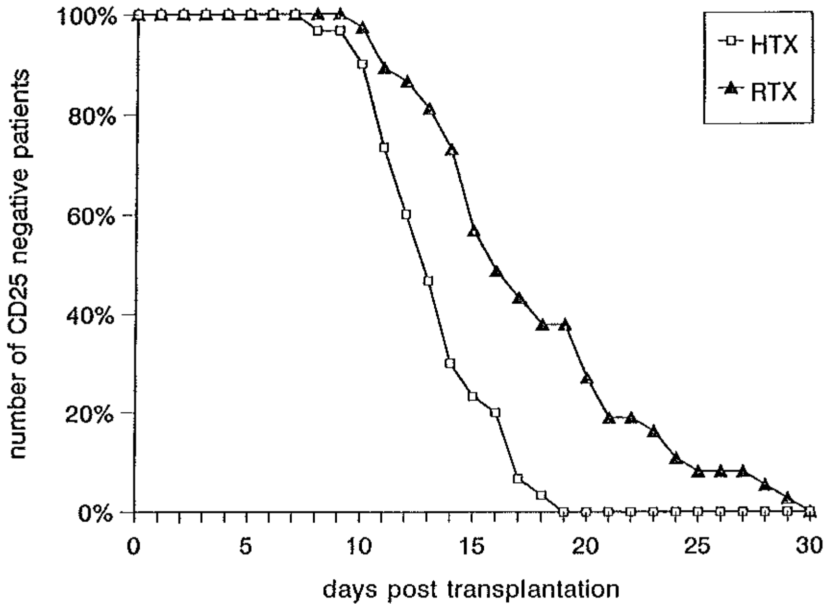

Figure 3.3 Plot of the percentage of heart transplant recipients (HTX, n = 30) and renal transplant recipients (RTX, n = 38) without free IL-2 binding sites (CD25) in peripheral blood cells in relation to the time after transplantation.

In most patients BT563 plasma levels returned to below the detection limit before free CD25 binding sites were detected in peripheral blood. However, in 8 of 55 patients the α-chain of the IL2-receptor on T-cells reappeared in the presence of a substantial level of circulating BT563 (arbitrarily chosen as >100 ng/ml). With this arbitrary value, in heart transplant recipients significantly more often positive $CD25^+$ cell counts coincided with positive BT563 plasma levels (7 out of 25 patients) compared with renal transplant recipients (1 out of 30 patients), $p = 0.0175$, Table I. The distribution of these heart transplant patients over the group of rejectors (2/14) and non-rejectors (5/16) proved to be not significantly different ($p = 0.3992$).

Table I. Recurrence of uncoated $CD25^+$ T-cells in the presence of detectable BT563 plasma levels ($> 100\ \mu g/L$)

	number of patients	days posttransplantation
kidney transplants	1/30	23
heart transplants[a]	7/25	12, 13, 13, 13, 17, 19, 19

[a] 5 patients were not included because incompleteness of data.

Generation of anti-mouse antibodies.
A wide range of pre-existing HAMA levels (1-42 mg/L IgG antibodies, 10-600 AU IgM antibodies) was found in these patients. Therefore, to express the induction of human anti mouse antibodies after BT563 and OKT3 therapy, the relative increases of the antibody concentration and not the absolute antibody levels were used (Table II). Values over four times the pretransplant or day zero value were sufficient dissimilar to consider as a positive HAMA reaction. No difference in the number of patients with a positive HAMA response of IgM or IgG antibodies was found between the heart and kidney transplant recipients in spite of the longer administration period in the kidney recipients and the higher

level of immunosuppression in the heart transplant patients. The IgM response became positive between day 9 and day 18 (median at day 13) in the BT563 treated heart transplant recipients and between day 6 and 59 (median 18 days) for the kidney transplant group.
In almost all BT563 treated patients the HAMA binding to BT563 (IgG_1) and OKT3 (IgG_{2a}) coated microtiterplates demonstrated an identical ratio in time after transplantation (results not shown). In only 4 patients a clear variation of this ratio in time after administration of BT563 was found which suggests an extra contribution of isotype and/or idiotype specific antibodies.
An increase of IgM HAMA could result in increased removal of circulating BT563 and in occurrence of acute rejection in heart or kidney transplant recipients. For heart recipients however the opposite relation was found after 10 days and less after 17 days, probably because of extra immunosuppression (fig 4). Considering in the first 2 months an increase of IgM HAMA concentration with a factor 4 as positive, we found a positive score in 7 out of 14 heart transplant patients with a rejection within one week after transplantation, in 5 out of 11 patients with a rejection more than one week after transplantation and in 4 out of 5 patients without any rejection during the period studied.

In 17% of the heart and kidney transplant recipients positive IgG antimurine responses were found after 15 to 47 days. All these patients also showed a positive IgM HAMA response but the clinical picture of these patients was not different from the patients without IgG response. Two patients with positive IgM and IgG responses were found within the group of fourteen heart transplant rejectors and the other two were found within the sixteen nonrejectors.

BT563 versus OKT3.

In the heart transplant patients receiving 5 mg/day OKT3 the maximal mAb plasma levels achieved (950 ± 80 ng/ml at day 6) and the mAb half-life ($T_{½}$ = 21 ± 2 hr) were substantially lower compared with the group of patients receiving 10 mg/day BT563 (previous section, fig 3.1). In 2 out of 20 patients OKT3 levels decreased already before the end of the administration period

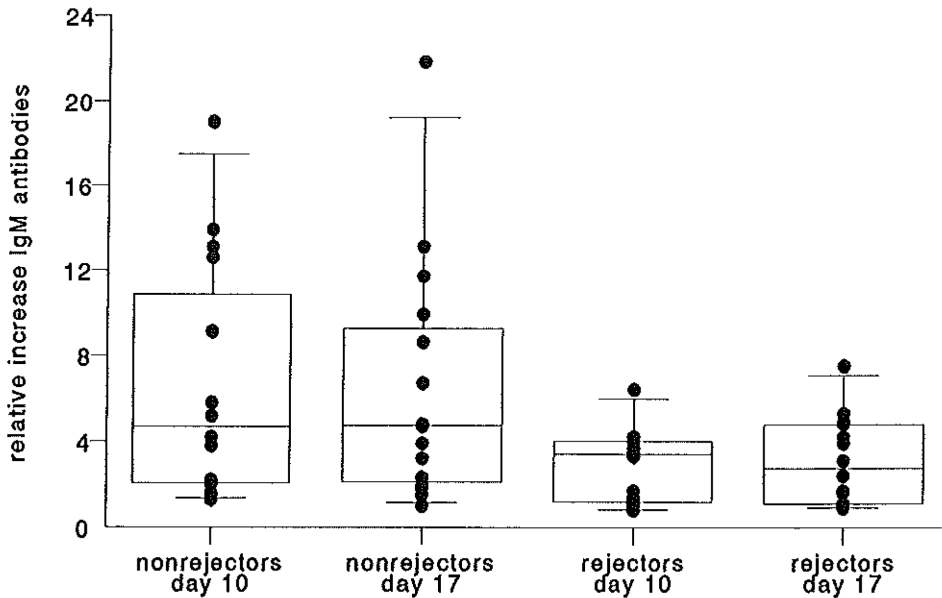

Figure 3.4 Relative increase of IgM HAMA at 10 and 17 days after heart transplantation in rejectors (n = 14) and non-rejectors (n = 16). Comparison of rejectors and non-rejectors at day 10 (p = 0.0340) and at day 17 (p = 0.0674).

coincident with increased IgM and IgG HAMA levels.
The effect of OKT3 administration is a strong reduction of all subsets of T-lymphocytes in the peripheral blood in contrast to the effect of BT563 (fig 5). TCRαß$^+$ (WT31$^+$) cells already reappeared at 5 days (mean, range 3-9 days) after finishing OKT3 administration. In BT563 treated patients the CD25$^+$ cells reappeared on a mean of 10 days (range 5-22 days) after the end of treatment.

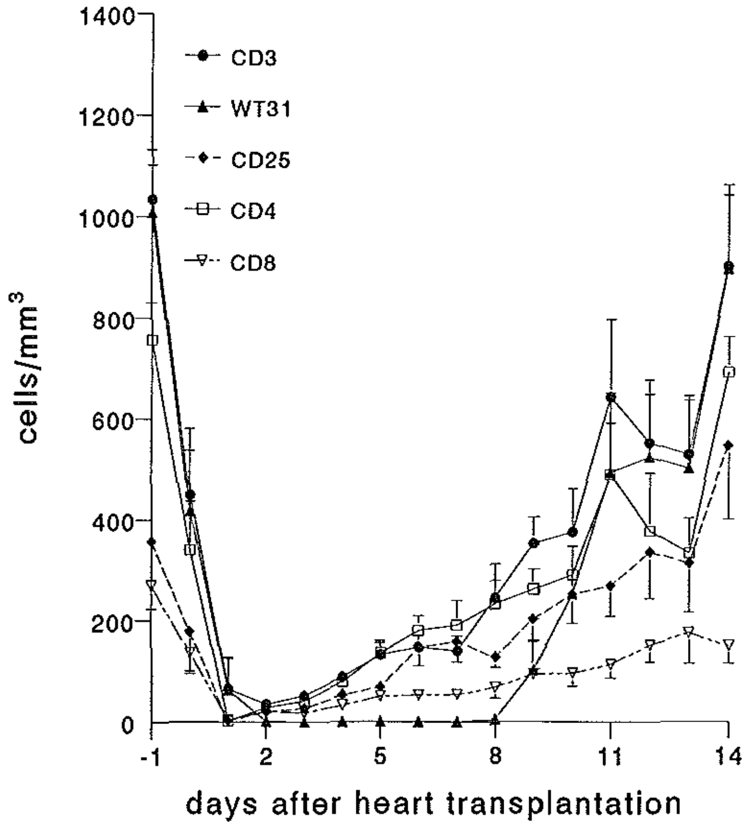

Figure 3.5 Effect of OKT3 on the number of T-cells and T-cell subsets in heart transplant recipients (n=20, mean ± SEM). After initial depletion T-cells returned modulated for the T-cell receptor (CD3 positive and WT31 negative lymphocytes).

No difference was found between the BT563 and OKT3 treated heart transplant patients with respect to the number of patients with a positive HAMA response of IgM antibodies (Table II). However, the IgM response was detected earlier after OKT3 than after BT563 treatment. In 55% of the patients treated with OKT3 an IgM response was found in the first sample tested (day 5-7 post

transplantation). Also the IgG response was earlier in the OKT3 treated group and became positive between day 7 to 21 (median day 14). For IgG antibodies, in contrast to IgM antibodies, the number of responders was higher in the OKT3 group (55%) compared with the BT563 heart transplant (13%, $p = 0.0036$) and kidney transplant recipients (21%, $p = 0.0174$). Nine OKT3 treated patients showed an relatively increased IgG HAMA response measured on OKT3 coated microtiterplates compared to BT563 coated plates which indicates an idiotype and/or murine IgG2a isotype specific reaction.
A high disappearance rate of the mAbs (OKT3 as well as BT563) in the plasma of the individual patient (a low $T_{½}$) was not related with the IgM or IgG response. (results not shown).

Table II. HAMA response after monoclonal antibody (BT563 or OKT3) treatment.

	IgM antibody response		IgG antibody response	
organ and treatment	negative	positive[a]	negative	positive[a]
RTX BT563	12	17	23	6
HTX BT563	14	16	26	4
HTX OKT3	8	12	9	11

[a] positive is an increase over 4 times the pretransplant value.

Soluble IL-2 receptor.

In one patient treated with BT563 the sIL-2 receptor concentrations in plasma were measured at regular intervals up to 60 days after transplantation (fig 6). During BT563 treatment there was a reduction of sIL-2R followed by a clear increase in the period thereafter. This patient generated also a HAMA IgM

response which was maximal around day 20 (8 times pretransplant level) and a HAMA IgG response between 35 and 60 days. The HAMA IgG concentration increased from 11 mg/L to 63 mg/L at day 41. Addition of BT563 to a sIL-2R standard sample reduced the measured value illustrating that circulating BT563 can influence the measured sIL-2R plasma levels.

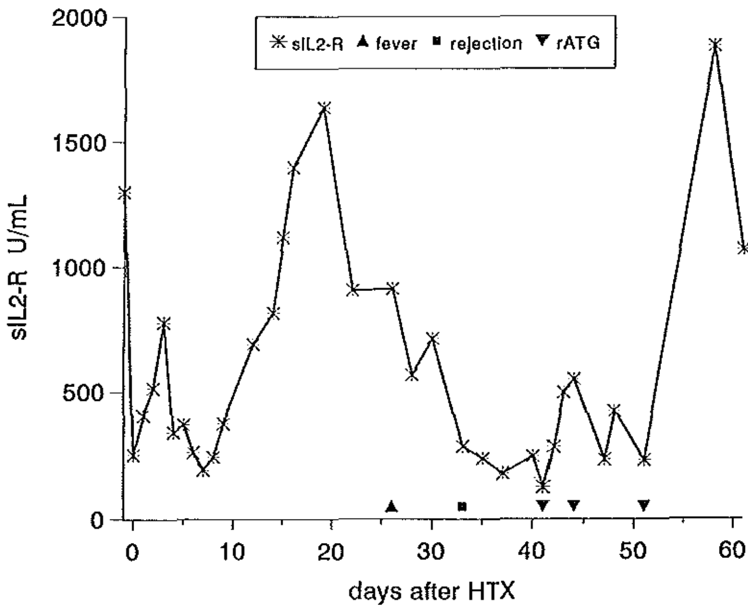

Figure 3.6 Soluble IL-2 receptor in plasma in a single patient after heart transplantation. The patient received 7 doses of 10 mg BT563 (days 0-6). Biopsy proven rejection was treated with rabbit anti-thymocyte globulin (rATG).

3.5 DISCUSSION.

The BT563 doses given proved to be very effective in coating the IL-2R α-chain on T-cells during the administration interval and a period thereafter. The free IL-2R α-chain on T-cells was reduced below the detection level[12]. Cellbound BT563 could be demonstrated with a PE-labeled rat anti-mouse antibody. In 8 patients (7 heart transplant recipients and only 1 renal transplant recipient) the IL-2α chain became detectable on T-cells concurrent with a substantial level of BT563 in the circulation, however. This means that in the heart transplant group, in spite of a shorter course of BT563 but with plasma levels during the adminstration period comparable with the kidney transplant group, the monoclonal antibody levels are not sufficient to suppress the reappearance of free α-chains of the IL2-receptor on the circulating blood cells in the presence of the mAb in a number of cases. The plasma levels obtained are far above the levels needed for inhibition of T-cell proliferation in vitro. We have demonstrated a maximal inhibition of lymphocyte proliferation in the mixed lymphocyte reaction already at BT563 levels of 500 ng/ml[12]. This inability to prevent the reappearance of CD25 was not directly related to the generation of antimurine antibodies as only 2 of these 7 heart transplant patients had an increased IgM response over 4 times the pretransplant level. Immune complex formation can explain this phenomenon in the renal transplant recipient. In this patient an abrupt increase of IgG HAMA was found at day 14, while the highest IgM HAMA titre were found between day 23 and day 28. BT563 was still detectable in the plasma (600 ng/ml) at day 23 together with 36% $CD25^+$ T-cells.

A mean distribution volume of 3.0 litre in kidney recipients and 2.6 litre in heart recipients was calculated based on the daily gift of 10 mg BT563 and the resulting mean plasma levels measured 24 hours thereafter. With a daily dose of 5 mg OKT3 a distribution volume of 4.1 litre was calculated. This indicates that most of the antibodies stay in the circulation and that the tissue concentration may be low. Because BT563 is a coating and not a modulating or a depleting mAb, cells infiltrating the graft may generate the free IL-2 receptor quickly on the cell membrane, resulting in intragraft lymphocyte proliferation. Indeed, in endomyocardial biopsies protocollary taken within the BT563 treatment period (at day 7) we found uncoated $CD25^+$ T-cells after immunohistological

staining[14].
After the last gift the plasma levels dropped in a few days below the detection level as a result of the high disappearance rate of both mAbs BT563 and OKT3. Short half-lives of mAbs in the circulation, (21-42 hrs) compared to 21 days for human IgGs, were also mentioned by others[15 16 17]. The plasma half-life of the mAbs will be influenced by the epitope density on the target cells, the number of cells carrying that epitope, and the regeneration of the epitope on these cells. The higher density per cell and the presence of the CD3 determinant on more cells compared to the p55 chain of the IL2-receptor may explain the difference in half-life observed between OKT3 and BT563. A reasonable explanation for the short half-life compared with human IgG is the existence of natural antimouse antibodies. Galili has described the expression of an α-galactosyl carbohydrate epitope on cells of nonprimate mammals and the presence of naturally occurring anti-Galα1-3Gal antibodies in the human population[18 19]. Borrebaeck and co-workers proved the existence of this glycosylation of mouse monoclonal antibodies to a degree which is mAb dependent[20]. The presence of these natural antibodies in human serum results in a quick removal of the mouse mAbs. Indeed mouse/human chimeric antibodies and humanized monoclonals have a longer in vivo survival, are substantially less immunogenic than their murine counterpart, and have a better efficacy as shown in primate cardiac allograft survival[15] and treatment of colon cancer in humans[21].
The reason why a high number of rejections in the first period of treatment with BT563 in the heart transplant group was found compared to the kidney transplant group, in spite of similar BT563 levels and similar HAMA generation, can possibly be the difference in the immunosuppressive protocols for both groups of patients. The additional gift of CsA immediately after transplantation, as done in kidney transplantation, is perhaps required for an effective block of the activation of the T-cells.
The BT563 treated patients have a low incidence of IgG antibody induction compared with the patients treated with OKT3 for the same period. This could be caused by a functional effect of BT563 on T- and B-cells and the signal needed by B-cells to switch from IgM to IgG antibody production. The fact that treatment with other α-IL-2R mAbs (33B3.1 and anti-Tac, both IgG_{2a}) results in a

strong response of neutralizing IgM and IgG antibodies favours another explanation, however. Soulillou[6] reported the generation of IgM and IgM antibodies in 80% of the patients one month after treatment with mAb 33B3.1. Similarly treatment with anti-Tac resulted in anti-murine antibodies in more than 60% of the patients[7]. Maybe differences in the subclasses of the mAbs (IgG_1 versus IgG_{2a}) can account for the different immune response. Three major classes of receptors for IgG on human leucocytes, hFcγRI, hFcγRII and hFcγRIII, are currently recognized which possess different affinities to the separate human and mouse IgG isotypes[22]. For the hFcγRII expressed on B-cells the affinities for murine IgG isotypes were found as $1>2>>>2a,3$. This hFcγRII has an important function in regulating B-cell activation through membrane immunoglobulin. Crosslinking of this hFcγRII with membrane immunoglobulin by murine IgG would deliver a dominant negative signal to B-cells leading to inhibition of both proliferation and production[23]. Maybe this inhibition is responsible for the low induction of IgG HAMA titre with BT563 (IgG_1).
In a pilot experiment in a single patient the sIL-2R concentrations in plasma during and after BT563 therapy were followed. Unfortunately the measurement of sIL-2R plasma levels are negatively biased by circulating BT563. The formation of HAMA can also influence the test results resulting in apparent high values as found between day 15 and 30. To avoid these influences the test has to be modified by using antibodies of a different species or adding irrelevant murine immunoglobulin and changing the incubation protocol.
In conclusion, BT563 therapy results in coating of $CD25^+$ T cells, while inducing low levels of IgG antibodies in only 17% of the patients. It is therefore useful in induction of immunosuppression if given together with cyclosporin. Results of peripheral monitoring of mAb plasma levels, HAMA responses and number of CD25 positive cells were not related with the clinical results. The low levels of induction of IgG HAMA's allows future reuse of the mAb BT563.

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Chapter 4

A randomized trial comparing safety and efficacy of OKT3 and a monoclonal anti Interleukin-2Receptor antibody (BT563) in the prevention of acute rejection after heart transplantation.

This chapter is based on an article with the same title, accepted for publication in Transplantation, by Gelder van T, Balk AHMM, Jonkman F, Zietse R, Zondervan P, Hesse CJ, Vaessen LMB, Mochtar LMB, Weimar W.

4.1 Abstract

In a prospective randomized trial BT563, a murine IgG_1 anti Interleukin-2Receptor antibody, was compared with OKT3 as early rejection prophylaxis after heart transplantation. Patients received either BT563 (n = 31) or OKT3 (n = 29) during the first 7 days after transplantation; cyclosporin was started on day 3. Median follow-up is 34 months.

A cytokine release syndrome occurred in the majority of patients of the OKT3 treated group but in none of the BT563 recipients. The mean duration of electrical stimulation of the heart in the BT563 group was longer than in the OKT3 group (5.1 vs 2.1 days). In both groups insertion of a permanent pacemaker was neccessary in one patient.

Freedom from acute rejection at 3 months was not significantly different between the 2 groups (BT563: 5/29 = 17% ; OKT3: 6/29 = 21%). In the BT563 group however, rejection tended to occur earlier after transplantation. There was no difference in the overall incidence of rejection. Infectious complications were evenly distributed in both groups. Malignancies occurred in two patients, both in the OKT3 group.

In conclusion, the use of this anti IL-2R MoAb in heart transplant recipients is safe and devoid of the side effects that accompany the use of OKT3. OKT3 and BT563 result in a similar freedom from rejection at three and twelve months after heart transplantation.

4.2 Introduction

The murine monoclonal anti-CD3 antibody OKT3 exerts its in vivo immunosuppressive effects by inducing major peripheral T cell depletion as well as antigenic modulation of the T-cell receptor complex[1]. Although effective as anti-rejection therapy and possibly as rejection prophylaxis in heart and kidney transplantation[2] [3], the administration of OKT3 is accompanied by a cytokine release syndrome[4] and by procoagulant effects[5] that may result in intragraft thrombosis and graft loss. The OKT3-induced profound and aspecific immunosuppression can be accompanied by an increased incidence of infectious complications[6] and by the development of malignancies[7]. Monoclonal antibodies

directed at activated lymphocytes only, lead to a more specific immunosuppression and might therefore circumvent these problems.
In contrast to resting T-cells activated T-cells, involved in allograft-rejection, express high-affinity Interleukin-2Receptors (IL-2R)[8]. The use of monoclonal antibodies directed against the IL-2R (IL-2R MoAbs) proved to result in prolonged graft survival in animal models[9 10]. Thereafter clinical trials with several different IL-2R MoAbs were performed in the prevention of acute rejection after kidney[11 12 13], combined kidney and pancreas[14] and liver[15 16] transplantation. Here we present the results of a controlled trial with BT563, a murine IgG_1-antiIL-2R MoAb, compared with OKT3 as early prophylaxis against rejection after heart transplantation.

4.3 Patients and Methods

Between November 1991 and February 1994 sixty consecutive recipients of a cardiac allograft were randomized for prophylactic anti-rejection treatment with BT563 or OKT3 after returning from the operation theatre. BT563 (Biotest Lab.-Dreieich Germany) was given in a dosage of 10 mg i.v. from day 0 to day 6 after transplantation. OKT3, 5 mg iv daily, was given following the same protocol. In addition to BT563 or OKT3 patients were treated with prednisone 50 mg daily from day 0, gradually decreasing to 15 mg at 4 weeks after transplantation. Cyclosporin was started at day 3 in a dosage of 8 mg/kg/day, aiming at whole blood trough levels of 400 - 500 ng/ml (radioimmunoassay). When the thrombocyte-count was above 100 . 10^9/ml, 50 mg azathioprine was given for the first 6 postoperative days in an attempt to prevent the formation of anti-murine antibodies[17]. Endomyocardial biopsies were performed weekly during the first 6 weeks, biweekly during the next month and monthly for the next four months after transplantation. Biopsies were repeated more frequently after a rejection episode.

Peripheral blood monitoring.

Before, during and after BT563 treatment peripheral blood samples were collected daily 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). Prior to incubation with MoAb, 100 μl whole blood was washed twice with 2 ml phosphate buffered saline 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 controle; CD3-FITC/CD19-PE; CD3-FITC/CD8-PE; CD3-FITC/CD4-PE; CD4-FITC/HLA-DR-PE, all obtained from Immunotech (Marseille, France); and the combination WT31-FITC (anti-TCRα/ß) /CD25-PE (MoAb 2A3) both obtained from Becton & Dickinson (San Jose, California). After 30 minutes incubation at room temperature, red blood cells were lysed with FACS lysing solution (Becton & Dickinson) during 10 minutes. 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. 1000 to 2000 lymphocyte events were acquired from each tube.
The CD25 MoAb 2A3 is also directed against the IL-2R α-chain, and is competitive with BT563[18]. In a number of patients we also analysed the presence of the CD122 (IL-2R ß-chain (p75)) on the T-lymphocytes by the MoAb combination TIC-1-FITC (Endogen, Boston, MA) / CD3-PE (Immunotech). A PE-conjugated polyclonal rat-anti-mouse antibody (RAM) (B&D) was used to determine whether CD25-positive cells in PBL were coated with BT563 during treatment.

Plasma BT563 levels.
BT563 trough levels were measured daily during treatment. Plasma levels were determined with an ELISA sandwich method using goat antimouse-IgG (Southern Biological Associates, Birmingham, AL, USA) and alkaline-phosphatase-(AP)-conjugated goat antimouse-IgG (H + L) antibodies (Jackson Immuno Research, West Grove, PA, USA). 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 TBS/Tw/BSA.

Anti BT563-antibodies.
Anti BT563-antibodies were measured in an ELISA system using microtiterplates precoated with 0.1 ml BT563 solution (3 μg/ml Phosphate Buffered Saline (PBS), pH 7.4). Plasma samples were initially 1:50 diluted and further in double dilution steps of 0.1 ml PBS. Human antibodies were detected with an AP-conjugated goat

antihuman-IgG antibody (Jackson Immuno Research) and quantified by comparison with two different reference plasma samples. The concentration of antimouse-IgG antibodies in these reference plasma's was 54 and 154 μg/ml respectively.

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[19] (Table 4.1). Biopsies graded 3A or higher were regarded to represent rejection episodes neccessitating anti-rejection treatment. In the majority of cases rejection was treated with 1 gram methylprednisolone (Solu-Medrol) i.v. on three consequetive days. Frequently recurring or refractory rejection episodes were treated with rabbit-ATG (RIVM, Bilthoven, The Netherlands). Recurrent episodes of steroid-unresponsive rejection after treatment with r-ATG were treated with a course of OKT3.

Table 4.1 Criteria for standardized cardiac biopsy grading. (J Heart Transpl 1990;9:587-593)

Grade	Histology
0	no rejection
1A	focal infiltrate without necrosis
1B	diffuse but sparse infiltrate without necrosis
2	one focus only with aggressive infiltration and/or focal myocyte damage
3A	multifocal aggressive infiltrates and/or myocyte damage
3B	diffuse inflammatory process with necrosis
4	diffuse aggressive polymorphous infiltrate / edema / hemorrhage / vasculitis / necrosis

Electrical stimulation of the heart.

In the immediate postoperative period routine temporary epicardial atrial and ventricular electrodes were used in all patients. Further chronotropic support was provided by isoprotenerol-infusion. The heart rate was kept above 110 bpm during the first post-operative days. Depending on the hemodynamic condition the heart rate was allowed to decrease gradually below 100 bpm by lowering the electrical stimulation rate and the isoprotenerol dose.

Statistics.

For the determination of levels of statistical significance two sided p values using Fisher's Exact Test were calculated.

4.4 Results

The baseline characteristics of the 60 patients that were entered into this study are shown in Table 4.2. The BT563 group and the OKT3 group were comparable in respect to all of these characteristics. The majority of recipients were male (82%), above 40 years of age (85%) and suffered from ischemic heart disease (65%). Most donors were below 40 years of age (83%). Donors and recipients were not prospectively matched for CMV-serology. CMV positive donor / CMV negative recipient combinations were equally distributed among the two groups (Table 4.2). Within hours after the first gift of BT563 the IL-2R bearing cells (CD25) in peripheral blood were coated with the antibody. Peripheral blood lymphocytes remained negative for free CD25 molecules for the duration of BT563 adminstration and for a period of 1 to 12 days (median = 4 days) thereafter. BT563 trough levels reached a plateau phase after day 4, with trough levels of 5300 (±200) 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 hrs.

Table 4.2 Baseline characteristics of heart transplant recipients

		BT563 (n=31)	OKT3 (n=29)
Median age	(range)	53 (14-65)	51 (24-65)
Median weight	(range)	69 (34-114)	63 (45-89)
Gender (M/F)		25/6	24/5
Underlying heart	disease (n)		
	ischemic	20	19
	cardiomyopathy	7	9
	valvular	4	1
Median donor age	(range)	27 (14-45)	27 (12-44)
Mismatch -HLA-(n)	A	1.3	1.2
	B	1.5	1.6
	DR	1.5	1.5
CMV mismatch (rec-don+)		7	6
Cold ischemia time (min)	median	155	152
	range	107-260	85-240
Preoperative amiodarione treatment		11	4

In the OKT3 treated group the cytokine release syndrome occurred in the majority of patients (23/29 fever, 6/29 hypotension, 8/29 rash, 19/29 altered mental status). None of the BT563 treated patients developed the typical cytokine release syndrome, although in some low-grade fever (10/31) and psychosyndrome (7/31) were found during the first postoperative days. Postoperative confusion after heart surgery is not unusual[20]. Haloperidol, used to treat signs of organic psychosyndrome (mostly visual and/or auditory hallucinations), was given to 7/31 (23%) of BT563 and to 19/29 (66%) of OKT3 treated patients.

In the BT563-group two patients died, one as a result of hemorrhagic stroke on day 4, the other due to sepsis after several thoracotomies for tamponade on day 17. These two patients were excluded from the analysis of rejection-incidence. Table 4.3 and Figure 4.1 show the numbers of patients free from rejection at several intervals after transplantation. In the BT563 group 13/29 (45 %) patients had their first rejection at week 1, whereas 15/29 (52 %) patients in the OKT3 group rejected between weeks 3 and 5. No differences between the two study groups were found for the total number of rejection episodes in the first year (2.8 vs 2.5 for the BT563 and OKT3 group respectively). Also no difference was found in the numbers of patients showing more than 2 rejection episodes (18/29 in BT563 and 15/29 in OKT3 recipients). At 3 months after transplantation freedom from rejection between the two groups was not significantly different. Treatment of the first rejection episode was successful with steroids in 15/25 (60 %) of BT563 and 16/24 (67 %) of OKT3 treated patients. The other 10/25 and 8/24 patients needed rATG for refractory rejection.

Table 2. Freedom from rejection

Weeks after Tx	1	2	3	4	5	6	12	52
BT563 (n=29)	16/29 (55%)	15/29 (52%)	12/29 (41%)	10/29 (34%)	9/29 (31%)	7/29 (24%)	5/29 (17%)	5/29 (17%)
OKT3 (n=29)	28/29 (97%)	25/29 (86%)	19/29 (66%)	16/29 (55%)	10/29 (34%)	9/29 (31%)	6/29 (21%)	5/29 (17%)

Table 4.3 Freedom from rejection after heart transplantation.

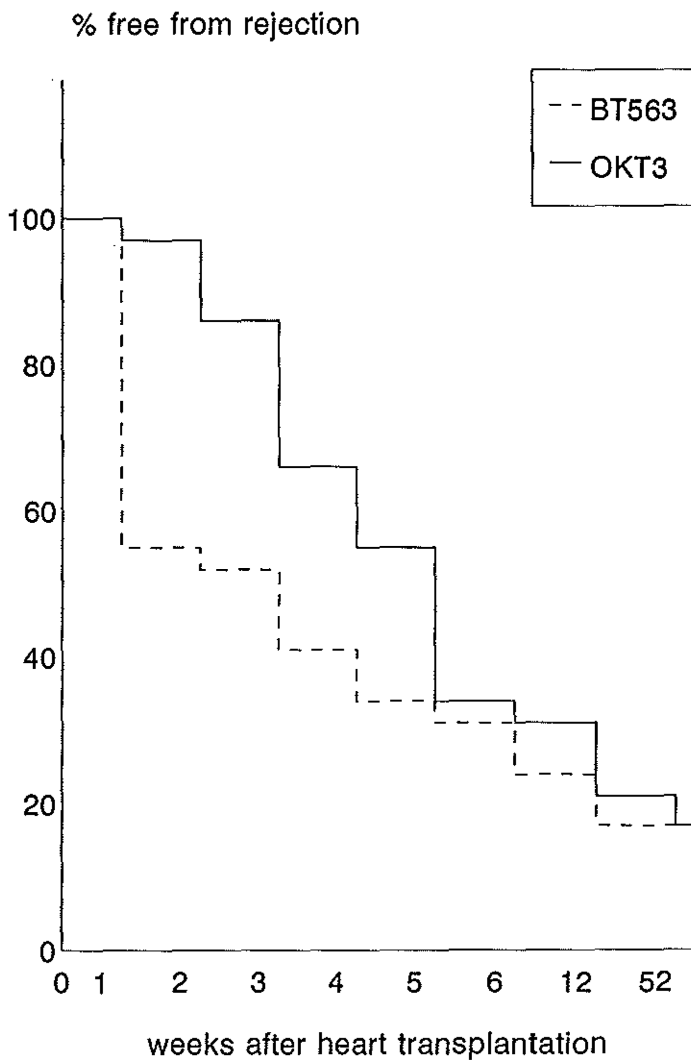

Figure 4.1 Freedom from rejection after heart transplantation, using induction immunosuppression with BT563 or OKT3.

Infectious complications within 90 days after transplantation are shown in Table 4.4. Most infections were of minor clinical significance. The herpes hominis virus infections were labial infections (blisters). Urinary tract infections were mainly found in the first postoperative week, probably as a result of bladder catheterization. Of the 10 mild respiratory tract infections in the BT563 group 5 occurred in two patients.

Table 4.4 Infectious disease after heart transplantation (≤90 days after tx)

	BT563	OKT3
CMV disease	3	1
Herpes Hominis Virus	7	14
Urinary tract infections	7	7
Respiratory tract infections	10	2
Intracerebral toxoplasmosis	1	0
Peridiverticular abces/perforation	2	0
Other infections	5	3

In the OKT3 group a mediastinal malignant lymphoma was diagnosed 5 months after transplantation in one patient, for which chemotherapy (CHOP) followed by radiotherapy was started, resulting in complete remission of the tumor and survival of the patient to date (now 32 months after transplantation). In another OKT3 treated patient a metastasized bronchuscarcinoma was found 23 months after transplantation. He died 8 months later.

Although temporary pacemaker electrodes are routinely placed in all heart transplant recipients, we found differences in pacemaker support between the two groups. After excluding two patients (one patient in both groups) who needed a permanent pacemaker, and the two patients in the BT563 group that died in the post-operative period, the mean duration of pacemaker support in the BT563 group was 5.1 days and in the OKT3 group 2.1 days. Pacemaker support for more than three days was needed in 11/28(39%) of BT563 and 4/28 (14%) of OKT3 treated patients ($p=0.07$). Although the difference did not reach statistical significance pre-operative amiodarone treatment was more frequent in the BT563 group (11/31) than in the OKT3 group (4/29) ($p=0.07$). Within the BT563 group pre-operative amiodarone treatment was also more prevalent in the patients needing pacemaker support for more than three days than in those who did not (7/11 vs 4/17; $p=0.05$). Of all patients needing pacemaker support for more than three days, only 4 (4/15) had an acute rejection in their first biopsy at week 1.

4.5 Discussion

In all patients receiving BT563 coating of all IL-2R positive cells was found in peripheral blood within one hour after the first gift. Coating persisted until 1 - 12 days (median 4 days) after the last dose. Despite this clear effect of BT563 on peripheral blood lymphocytes, endomyocardial biopsies taken at the end of the prophylactic treatment period showed acute rejection in 13/29 patients. Immunohistochemistry of the biopsies demonstrated the presence of cells bearing uncoated IL-2Receptors within these endomyocardial biopsies during acute rejection[21].

The cytokine release syndrome, known to be associated with the use of OKT3, did not occur in the BT563 treated patients. Anti IL-2R MoAbs as a group appear to be free from this potential life-threatening side-effect[11-16].

Freedom from rejection at 12 weeks after transplantation was not different between the OKT3 (6/29 = 21%) and BT563 (5/29 = 17%) treated group. However, as is clearly demonstrated in Table 4.3 and Figure 4.1, the first acute rejection in the BT563 treated patients was often found in the first or second biopsy, whereas

most first acute rejection episodes in the OKT3 group occurred in weeks 3, 4 or 5. There was no difference in the severity of these rejections, as is shown by the similarity of required anti-rejection treatment courses. In both groups about two thirds of first acute rejections were steroid-responsive. All steroid-unresponsive rejections were treated with anti-T cell therapy (rabbit-ATG or OKT3). After the first rejection episode many patients experienced one or more subsequent acute rejections (Table 4.5). Cumulative numbers of rejections nor the required rejection treatments differed significantly between the groups.

Table 4.5 Number of rejection episodes after heart transplantation.

no. of rejections	BT563	OKT3
0 - 1	8	10
2 - 3	11	11
>3	10	8

Infectious complications were not different between the two groups. Although many patients received anti T-cell therapy with ATG or OKT3 only four patients developed CMV disease, possibly because all CMV positive donor/CMV negative recipient combinations received prophylaxis with CMV hyperimmunoglobulins[22]. The sample size of this study does not allow conclusions regarding malignancy induction, but it is remarkable that the two patients with malignancies both received OKT3-prophylaxis[23].

The difference in duration of required electrical allograft stimulation cannot be explained by a difference in cold ischemia time between the two groups. Treatment with amiodarone prior to transplantation was more frequent in BT563 (11/31) compared to OKT3 recipients (4/29) ($p=0.07$) and is the most likely explanation for the prolonged pacemaker support[24]. No relation was found between prolonged pacemaker support and the occurrence of early acute rejection. The two permanent pacemakers were surgical complications, caused by intra-operative lesions and suturelines.

Although the low incidence of short and long term side effects observed are an advantage of BT563, the occurrence of early rejections in half of the BT563 treated patients is disappointing. A more favourable result with this monoclonal was obtained in a double-blind trial of rejection prophylaxis in renal transplant recipients, comparing a 10-day course of BT563 to placebo[25]. In that trial a significant reduction of early acute rejections was found in the BT563 treated patients. Within the first 4 weeks after transplantation only 1/27 BT563 treated patients experienced an acute rejection, compared to 7/29 of the patients in the placebo-group ($p = 0.05$). In contrast to the study presented here all renal allograft recipients had started cyclosporin on day 0. In animal studies a synergistic effect of the combination of cyclosporin and anti IL-2R MoAbs has been demonstrated[26 27]. In our heart transplant recipients we started cyclosporin at day 3 after transplant in order to prevent renal insufficiency in the immediate postoperative period. One may hypothesize whether the results of rejection-prophylaxis with BT563 in these patients would have been better when cyclosporin had been initiated on the day of transplantation, thereby making optimal use of the synergistic effect of combining BT563 and cyclosporin. The results of our renal study support this hypothesis.

In conclusion, the use of this anti IL-2R MoAb in heart transplant recipients was associated with less side effects than OKT3. The freedom from acute rejection in BT563 and OKT3 treated patients at 3 and 12 months was not significantly different. However, in BT563 treated patients the interval to the first rejection is shorter.

4.6 References

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Chapter 5

Intragraft monitoring of rejection following prophylactic treatment with monoclonal anti Interleukin-2Receptor antibody (BT563) in heart transplant recipients.

This chapter is based on an article with the same title, published in The Journal of Heart and Lung Transplantation 1995;14:346-350, by Gelder van T, Mulder AH, Balk AHMM, Mochtar B, Hesse CJ, Baan CC, Vaessen LMB, Weimar W.

5.1 Abstract.

In an open randomized study OKT3 and BT563, a murine IgG_1 anti IL-2R MoAb, were given as rejection prophylaxis during the first week after heart transplantation. Cyclosporin therapy was initiated at the third postoperative day. In half of the BT563 treated patients an acute rejection was histologically demonstrated at week 1, while OKT3 treated heart transplant recipients had a rejection-incidence at week 1 of only 9%. In peripheral blood during BT563 treatment CD25-positive cells (i.e. cells bearing the IL-2R) were coated with the antibody. However, immunohistologic studies of endomyocardial biopsies taken one week after transplantation showed the presence of uncoated CD25-positive cells within these biopsies in 8/10 (80%) of rejecting patients. In patients without rejection uncoated CD25-positive cells were present in the biopsies of only 2/9 (22%) patients. RT-PCR-studies on biopsy-material showed the presence of mRNA for the IL-2R in all, and for IL-2 in 3/5 (60%) of biopsies of rejecting grafts. Other studies, in which anti IL-2R MoAbs and cyclosporin were simultaneously given from the start, did show a reduction in early rejection after organ transplantation. Our present results suggest that this synergistic effect can only be reached if anti IL-2R MoAbs are given in the presence of cyclosporin, thereby influencing different steps in the immune cascade.

5.2 Introduction.

Interleukin-2 (IL-2) and the IL-2Receptor (IL-2R) play an important role in the generation of a normal immune response[1]. Therefore, it was a logic step to develop monoclonal antibodies (MoAbs) directed against the IL-2R in order to improve allograft survival[2]. Used as prophylaxis against early rejection these agents were considered to be valuable in kidney allograft recipients[3 4]. Anti IL-2R MoAbs have also been used succesfully in the prevention of rejection of cardiac allografts in several animal models[5 6]. In November 1991 we started an open randomized, controlled trial of BT563 (a murine IgG_1 anti IL-2R MoAb, directed against the α-chain (p55) of the IL-2R) compared with murine monoclonal CD3 antibody (OKT3) as rejection prophylaxis after heart transplantation.

We report data of the monitoring of peripheral blood, BT563-levels, anti-BT563 antibody formation, immunohistology and gene expression of IL-2 and IL-2R of endomyocardial biopsy (EMB) specimens and speculate on the possible causes of

the high rejection rate.

5.3 Methods

Cardiac allograft recipients were randomly assigned to receive prophylactic anti-rejection treatment with BT563 or OKT3 after returning from surgery. BT563 (Biotest Lab.- Dreieich Germany) was given in a dosage of 10 mg intravenously from day 0 to day 6 after transplantation. The control group received 5 mg OKT3 according to the same protocol. In addition to BT563 or OKT3 from day 0 patients were treated with prednisone 50 mg daily, gradually decreased to 15 mg at 4 weeks after transplantation. Cyclosporine was started at day 3 in a dosage of 8 mg/kg/day, to achieve whole-blood levels of 400 to 500 ng/ml (radioimmunoassay). When the thrombocyte count was greater than 100 $.10^9$ cells/ml, 50 mg azathioprine was given for the first 6 postoperative days in an attempt to prevent the formation of anti-murine antibodies. EMB was performed weekly for the first 6 weeks after transplantation. This study was approved by the Hospital Ethical Committee and informed consent was obtained from all patients.

Peripheral blood monitoring.

Before, during and after BT563 treatment peripheral blood samples were collected daily in EDTA containing tubes and monitored for the presence of T cell subsets by two color flow-cytometry using MoAb directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). One hundred microliters whole blood was incubated with 20μl of the following dual MoAb combinations : CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2a}-PE as isotype controle; CD3-FITC/CD19-PE; CD3-FITC/CD8-PE; CD3-FITC/CD4-PE; CD4-FITC/HLA-DR-PE, all obtained from Immunotech (Marseille, France); and the combination WT31-FITC(anti-TCRα/ß)/CD25-PE(MoAb 2A3) both obtained from Becton & Dickinson (San Jose, California, USA). After 30 minutes incubation at room temperature, red blood cells were lysed with FACS lysing solution (Becton & Dickinson) for 10 minutes. 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 lymphocyte events were acquired from each tube.

The CD25 MoAb 2A3 is also directed against the IL-2R α-chain, and is competitive

with BT563[7]. A PE-conjugated polyclonal rat-antimouse antibody (RAM) (B&D) was used to determine whether CD25-positive cells in PBL were coated with BT563 during treatment.

Plasma BT563 levels.

BT563 trough levels were measured daily during treatment. Plasma BT563 levels were determined with an ELISA sandwich method with goat antimouse-IgG (Southern Biological Associates, Birmingham, AL, USA) and alkaline-phosphatase-(AP)-conjugated goat antimouse-IgG (H + L) antibodies (Jackson Immuno Research, West Grove, PA, USA). Plasma samples were diluted 1:450 and 1:2000 with a combination of tris(hydroxymethyl)aminomethane (TRIS)-buffered saline solution, polysorbate 20 (Tween 20), and 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 TBS/Tw/BSA.

Anti BT563-antibodies.

Anti BT563-antibodies were measured in an ELISA system with microtiterplates precoated with 0.1 ml BT563 solution (3 μg/ml, Phosphate Buffered Saline (PBS), pH 7.4). Plasma samples were initially diluted to 1:50 and further in 0.1 ml double dilution steps. Human antibodies were detected with an AP-conjugated goat antihuman-IgG antibody (Jackson Immuno Research) and quantified by comparison with two different reference plasma samples. The concentration of antimouse-IgG antibodies in these reference plasmas was 54 and 154 μg/ml respectively.

Pathology.

A pathologist, unaware of the immunosuppressive protocol of the patient, examined EMB- specimens stained with hematoxylin-eosin by light microscopy. These biopsies were graded according to the criteria of the International Society for Heart and Lung Transplantation[8]. Biopsies graded as 3A, 3B or 4 were regarded to represent acute rejection episodes necessitating antirejection treatment. Rejection was treated in the majority of cases with 1 gram methylprednisolon (Solu-Medrol) intravenously on three consecutive days. Refractory rejection was treated with rabbit-ATG.

Snap-frozen EMB specimens were cut in 5 μm sections, air dried and fixed in

aceton. The slides were subsequently incubated with the appropriately diluted monoclonal antibody (CD3 = anti-Leu-4; CD4 = anti-Leu-3a; CD8 = anti-Leu-2a; CD25 = 2A3 (B&D)). The slides were rinsed in PBS and incubated with biotinylated antimouse-immunoglobulins (1:50 in PBS; Biogenex, San Ramon, California), rinsed and labeled with AP-labeled streptavidin (Biogenex, 1:50 in PBS). The enzyme was finally detected with new fuchsin as a substrate as described by the manufacturer. The slides were examined and the number of positive staining cells was estimated. The results are expressed per half field at 100x magnification (obj. 100x). A total field at this magnification measures 2.54 mm^2. Five groups were delineated: 0, 1-10, 11-30, 31-100 and more than 100 positive cells per half field (1.27mm^2).

RNA extraction and transcription.

Snap-frozen EMB specimens, available from 11 patients, were homogenized using disposable micropestles (Merck/Eppendorf) after which 10^4 mouse 3T3 cells (American Type Culture Collection, Rockville, Md.) were added to improve yield. Total RNA was extracted from snap-frozen EMB using a modified version of the guanidinium method described by Chomczynski and Sacchi[9]. After extraction, RNA was precipitated and dissolved in 50 μl diethylpolycarbonate treated water (DEPC-H_2O). First-strand cDNA synthesis was performed at 42 °C for 90 min using 25 μl ectracted RNA, 2.5 μl Moloney murine leukaemia virus (MMLV) reverse transcriptase (200 U/μl; Gibco-BRL, Gaithersburg, MD) and 0.25 μg hexanucleotides (0.5 μg/μl; Promega Corporation, Madison, WI) in a total reaction volume of 50 μl.

PCR amplification and Southern Blot Analysis.

Subsequently, 5 μl cDNA was amplified in a thermal cycler using 2 U Taq DNA polymerase (Promega) and 50 pmol of 3' and 5' human sequence specific primers (Clontech Laboratories, Palo Alto, Calif.). These primers were located next to splice sites, to allow discrimination for RNA only. PCR primers detecting transcripts for the human keratin gene (sense primer 5'TGAAGATCCGTGACTGGTAC3' and antisense primer 5'ATGTCGGCTTCCACACTCAT3') were used as an internal control to confirm succesful RNA extraction and cDNA amplification. The amplification temperatures and times were : 95 °C for 5 minutes, followed by 40 cycles with a 1 minute 95 °C denaturation, a 2 minute 60 °C annealing of primers and a 3 minute 72 °C extension of primers. The last step was extended for 7

minutes. PCR products were electrophoresed on a 2% agarose gel in tris borate etylenediamnie tetraacetic acid, transferred to Hybond N^+ (Amersham, England) and hybridized with gamma^{32}P endlabeled internal probes, which are located across the splice-site (IL-2 and IL-2R α-chain: Clontech Lab. and keratin: 5'TGTCCTTCTGCAGATTGACAATGCCCGTCT3'). Hybridization was detected by autoradiography.

5.4 Results.

Treatment with BT563 was not accompanied by side effects, in contrast to OKT3 treatment, where the cytokine release syndrome (fever, confusion, hypotension) was seen in the majority (8/11) of patients. Before BT563 therapy was instituted 10% to 25% of the PBLs were positive for both the α and the ß-chain of the IL-2R, as detected by the MoAb 2A3 and anti-IL-2Rß respectively. After the first injection of BT563 PBLs did not stain with 2A3, whereas a positive staining with rat antimouse antibody was found. The relative numbers of WT31/CD3, CD3/CD4, CD3/CD8 and CD3/HLA-DR positive cells did not change, indicating that the IL-2R had been coated by the antibody. This situation persisted during BT563 administration and uncoated CD25-positive cells were not found in peripheral blood until at least 4 days after the last gift. In all patients in whom an acute rejection was shown in the biopsy specimen taken at the end of the BT563-treatment period, no uncoated CD25 positive cells could be detected in peripheral blood at the moment of rejection. BT563 levels were not significantly different between rejecting and non-rejecting patients (Table 5.1). At week 1 human antimouse antibodies (HAMA's) were found in only three of six nonrejecting and one of seven rejecting patients.

Table 1. Endomyocardial biopsies in heart transplant recipients.

Patient initials	Pathology Grade	BT563 levels day 6	Immunohistochemistry				mRNA expression		IgM-HAMA
			CD3	CD4	CD8	CD25	IL-2	IL2R	
HA	1A	5200	3	3	2	1			+
LI	1A	5700	0	0	0	0			-
FO	1A	3900	ND	ND	ND	ND			+
PE	0	5600	2	3	1	0			-
VW	2	6100	1	1	0	0			-
HU	0	5800	1	2	1	0	-	+	+
VF	1A	4700	1	3	1	1	-	+	
VR	0	3600	1	3	1	0	-	+	
VS	0	8500	1	2	1	0	-	+	
BO	0	6400	2	3	1	0	-	+	
DO	1A	6200	ND	ND	ND	ND	-	+	
KN	3A	4200	2	2	1	0			-
KE	3A	5600	ND	ND	ND	ND			+
HR	3A	1700	3	3	3	2			-
GO	3A	4300	2	3	1	1			-
ST	3A	4200	3	3	1	1			-
KA	3A	3700	1	3	2	2	+	+	-
BA	3A	6600	2	3	2	2	ND		-
SO	3A	4700	2	3	2	2	+	+	
GE	3A	8200	3	3	3	2	-	+	
BE	3A	6100	2	4	2	1	-	+	
RO	3A	6700	2	2	2	0	+	+	

Table 5.1 Endomyocardial biopsies in heart transplant recipients.
Legends. BT563 levels in ng/ml. Scoring of CD3, CD4, CD8 and CD25-positive cells according to numbers of cells per half field (obj. 100x): 0 = 0 cells; 1 = 1-10 cells; 2 = 11-30 cells; 3 = 31-100 cells; 4 = >100 cells). IgM-HAMA : positive if a more than fourfold increase in IgM-HAMA was found within the first 10 days after transplantation.

Endomyocardial biopsies taken after 1 week of prophylactic treatment with BT563, using HE-light microscopic examination, showed acute rejection in 11/22 (50%) patients (all Grade 3A according to the criteria of the International Society for Heart and Lung Transplantation) was found (Table 5.1). Immunohistologic studies showed the presence of uncoated CD25-positive cells in these biopsies in eight of ten (80%) patients (Table 5.1). In the patients without rejection there were uncoated CD25-positive cells in the biopsies of only two of nine (22%) patients. Apart from CD25-positive cells intragraft presence of CD3, CD4 and CD8-positive cells was also correlated with the occurrence of acute rejection (Table 5.1). No significant differences between rejecting patients and non-rejecting patients existed for HLA-matching, body weight or cyclosporin-levels. In the control-group of OKT3 treated patients at week 1 after transplantation the rejection incidence was one in eleven patients (9%).

Reverse transcriptase PCR studies were performed on 11 snap-frozen and stored EMBs. 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 IL-2 and IL-2R or keratin. mRNA for IL-2R was found to be present in all samples, regardless of whether a rejection was present. mRNA for IL-2 could not be demonstrated in six non-rejection EMBs, but was found in three of five (60%) of rejection-EMBs (Table 5.1).

5.5 Discussion.

Prophylactic administration of BT563 in cardiac allograft recipients during the first week after transplantation resulted in coating of CD25 (i.e. IL-2R α-chain) on peripheral blood lymphocytes. Endomyocardial biopsies taken after this first week showed acute rejection in 11 of 22 (50%) of BT563-treated patients. Using immunohistologic techniques uncoated CD25-positive cells were found to be present in a high percentage (80%) of the rejecting patients (Table 5.1). Despite the effect of BT563 on peripheral blood, this prophylactic administration of BT563 could not prevent intracardial lymphocyte activation and proliferation. PCR-studies on biopsy material also showed the presence of mRNA for the IL-2R in all, and for IL-2 in three of five (60%) biopsies of rejecting grafts (Table 5.1). We have

previously shown IL-2 gene expression in relation to acute cardiac rejection[10].

It is remarkable that we still found rejection of the graft even though results from peripheral blood looked so promising. As is clear from Table 5.1 the BT563 blood levels did not differ between rejecting and non-rejecting patients. Neither can HAMA-formation explain the difference between the 2 groups. Lymphocyte activation and proliferation in the graft nevertheless did occur and may have been the result of low intragraft BT563 levels. Alternatively lymphocyte activation may have taken place through non-IL-2R dependent pathways, resulting in a rejection-incidence of 50%.
In a prospective, double-blind, randomized, placebo-controlled study on the safety and efficacy of BT563 as rejection-prophylaxis in kidney transplantation we have found a significant effect of BT563 in the prevention of early rejection episodes[11]. Patients were treated with BT563 or placebo for the first 10 days, while cyclosporin was started six hours post-transplantation. In the present heart-transplantation study cyclosporin was started at the third post-operative day, in order to avoid the nephrotoxic effects of cyclosporine in the first post-operative days. Cyclosporin inhibits the expression of IL-2 at the transcriptional level[12], and possibly of the IL-2R as well[13]. In animal studies a synergistic effect of cyclosporin and anti IL-2R MoAbs has been demonstrated, probably as the net result of influencing different steps in the immune cascade[14 15]. In view of the significant effect on rejection prophylaxis found in the kidney and liver[16] transplantation studies, it may be that clinical rejection can be prevented only when anti IL-2R MoAb therapy is given in the presence of cyclosporin.

In summary, at week 1 after transplantation we have found a high incidence of rejection in heart transplant recipients who were prophylactically treated with BT563. Despite a clear effect of BT563 on peripheral blood, with coating of all IL-2R bearing cells, a considerable number of CD25-positive cells was found to be present within the grafts. An activated state of the IL-2 & IL-2R pathway was also shown with reverse transcriptase PCR-techniques. We postulate that the high failure rate of this prophylaxis is at least partly due to the fact that cyclosporin was only started at day 3 after transplantation ,thereby missing the synergistic effect of combining anti IL-2R MoAb and cyclosporin.

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Chapter 6

A double-blind, placebo-controlled study of monoclonal anti Interleukin-2Receptor antibody (BT563) administration to prevent acute rejection after kidney transplantation.

This chapter is based on an article with the same title, published in Transplantation 1995;60:248-252, by Gelder van T, Zietse R, Mulder AH, Yzermans JNM, Hesse CJ, Vaessen LMB, Weimar W.

6.1 Summary

In a double blind, randomized, placebo-controlled trial BT563, a murine IgG_1 anti-Interleukin-2Receptor antibody, was given as a rejection prophylaxis after kidney transplantation. Drug related side effects were not observed. During the 10-day course of BT563 no rejections (0/27) were found, whereas a rejection episode occurred in 7 patients (7/29) ($p=0.01$) during placebo treatment. Within the first 4 post-operative weeks freedom from rejection in the BT563 group and in the placebo-group was 96% vs 76% ($p=0.05$). Due to rejection in the placebo-group 2 grafts were lost. At three months an overall rejection-incidence in the BT563 and placebo-group was found of 3/27 (11%) vs 8/29 (28%) ($p=0.18$). Infectious complications were distributed equally between the 2 groups. CMV-disease, found in 3 placebo-treated patients, occurred after rejection-treatment (2/3). Within the BT563 group one patient lost his graft due to renal artery thrombosis, two grafts were lost as a result of technical failure and two patients had a squamous cell carcinoma that could be treated curatively. We conclude that the use of the anti IL-2R MoAb BT563 effectively prevents rejection after kidney transplantation without increasing infectious complications.

6.2 Introduction.

Interleukin-2 (IL-2) is a key cytokine in the generation and regulation of the immune response[1]. It is produced by activated T-cells and induces proliferation and differentiation of T- and B-lymphocytes. IL-2 is one of the strongest amplifiers of T-cell activation leading to acute cellular allograft rejection[2]. Resting T-cells express neither the α (p55) low-affinity component of the IL-2Receptor (IL-2R) nor the functional high-affinity IL-2R. Following antigenic stimulation through the T-cell receptor, specific T-cells are activated, IL-2 is secreted, and functional IL-2R is transiently expressed, allowing antigen-committed lymphocytes to expand[3]. The clinical use of IL-2R antagonists after transplantation may therefore result in a selective blockade of T-cell activation, thereby preventing rejection. Thus it may provide an elegant alternative to the anti pan-T-cell antibody preparations like the monoclonal antibody OKT3 and polyclonal antithymocyte and antilymphocyte globuline preparations, that react indiscriminately with both activated and resting

T-cells[4]. Overimmunosuppression, inherent to their usage, is reflected in the induction of infections and malignancies. In contrast to these aselective agents anti IL-2R antibodies may not be associated with increased risks of infections or malignancies. After having been used as a treatment for graft-versus-host disease in bone marrow transplantation[5], anti IL-2R monoclonal antibodies (MoAb) have been given as rejection-prophylaxis after kidney[6,7,8], combined kidney/pancreas[9] and liver[10,11,12] transplantation. It has been suggested that their efficacy was comparable to that of polyclonal anti T-cell preparations[6,12]. Disappointingly in the only available placebo-controlled study no beneficial effect of anti IL-2R antibody prophylaxis was observed after liver transplantation[10]. Monoclonal anti IL-2R antibodies may however differ vastly in their biological activity, due to differences in epitope recognition, affinity, isotype etc.

We have performed a double-blind, placebo-controlled study to prevent rejection after kidney transplantation, with an anti IL-2R antibody (BT563), that in vitro showed high efficacy in blocking the mixed lymphocyte reaction[13].

6.3 Material and Methods

BT563 is a murine antibody of the IgG_1-kappa isotype, directed against the p55-chain of the IL-2R complex. It was previously known as B-B10 and was developed and produced by Wijdenes in Besancon[5]. It is currently being produced by Biotest Pharma, Dreieich, Germany.

Patients

From March 1992 through August 1993 60 first renal allograft recipients were randomized for treatment with either BT563 or placebo. The study was approved by the Hospital Ethical Committee and written informed consent was obtained prior to randomization. All patients received pretransplant blood transfusions and had negative crossmatches with their donors in historical and current sera. Baseline characteristics of the patients are given in Table 6.1. The causes of end-stage renal failure were comparable. For all patients the 1-year follow-up results are shown.

Table 6.1. **Base-line characteristics of kidney allograft recipients.**

	Placebo n = 30	BT563 n = 30
age (yrs) - median (range)	45 (19-65)	43 (22-60)
sex M/F	19 / 11	18 / 12
race - Caucasian/other	25 / 5	27 / 3
weight (kg) - median (range)	75 (50-120)	68 (54-95)
dialysis prior to tx - CIHD	16	19
- CAPD	8	9
- none	6	2
cadaver donor/living related	22 / 8	25 / 5
donor age (yr) - median (range)	37 (12-64)	50 (15-71)
donor sex - M/F	18 / 12	16 / 14
mean no. mismatches HLA-A/B/DR	0.9 / 0.6 / 0.4	0.9 / 0.9 / 0.5
Panel Reactive Antibodies (<10%)	26	26
Cold Ischemia Time - median (range)	26 (12-37)	27 (13-36)

Immunosuppression

Two hours after surgery patients received two numbered ampules intravenously, each containing 5ml of either BT563 (1 mg/ml) or placebo (NaCl 0.9%). The next morning they received another 10 ml and this was continued for the first ten post-operative days. Cyclosporine (CsA) was given at a dose of 2 mg/kg/day i.v. until day 3, and at 8 mg/kg/day orally thereafter. Dosage adjustments were made aiming at a whole blood CsA-level of 300 ng/ml for the first 3 months. Prednisone was tapered from a dosage of 50 mg i.v. in the first 2 days to 15 mg orally from day 3.

Peripheral blood monitoring for T-cells.

Venous blood samples were obtained before transplantation, daily during the first 14 post-operative days, and twice weekly thereafter, until 1 month after transplantation, to monitor the presence of T cell subsets by two color flow cytometry using MoAb directly conjugated to fluorescein (FITC) or phycoerythrin (PE).

Whole blood (100μl) was added to Falcon 2052 tubes (Becton & Dickinson, Lincoln Park, New Jersey, USA), washed twice with 2 ml phosphate buffered saline, to remove BT563 and human anti-mouse antibodies that might interfere with the staining procedures. After the cells were pelleted by centrifugation, 20 μl of the following dual MoAb combinations were added: CD45-FITC/CD14-PE; IgG1-FITC/IgG2a-PE as isotype control; CD3-FITC/CD19-PE; CD3-FITC/CD8-PE; CD3-FITC/CD4-PE; CD3-FITC/HLA-DR-PE (all from Immunotech, Marseille, France); and the combination anti-TCR$\alpha\beta$-FITC(clone WT31)/CD25-PE(clone2A3) (both from B&D, San Jose, California, USA). For the detection of CD122 (the IL-2Rβ-chain (p75)) the cells were stained with 20 μl of the FITC conjugated MoAb TIC-1 (Endogen, Boston, USA). To screen for BT563 bound to T-cells because of treatment, 5 μl of a PE conjugated polyclonal Rat-anti-mouse (RAM) (B&D) was added to washed and unwashed whole blood cells. Both anti-CD122 and RAM were used in combination with CD3-FITC. After 30 minutes of incubation at room temperature, red blood cells were lysed with FACS lysing solution (B&D) during 10 minutes. After they were washed with sheet-fluid Cellpack (TOA Medical Electronics, Kobe, Japan) cells were analysed on a FACScan flowcytometer (B&D) using SimulSet software (B&D) for data analysis. To establish an analysis gate that included 95% lymphocytes, the CD45/CD14 reagent was used. From each tube 1000-2000 lymphocyte events were acquired. For the detection of free binding sites on the IL-2R (CD25) positive cells the MoAb 2A3 is suitable, since, like BT563, 2A3 is directed against the IL-2Rα-chain, and recognizes an epitope of the same cluster on the p55-chain[13]. Total white blood cell count was performed on a Sysmex microcell counter (TOA) after lysing the red blood cells with Quicklyser QLY-200A (TOA).

Monitoring for BT563.
Plasma BT563 levels were determined with an ELISA sandwich method using goat antimouse-IgG (Southern Biological Associates, Birmingham, AL, USA) and alkaline phosphatase-(AP)-conjugated antimouse-IgG (H + L) antibodies (Jackson Immuno Research, West Grove, PA,USA). Wells were filled with 8 serial dilutions of human plasma samples or reference samples, starting at a 1:50 dilution, and quantified by comparison of calculated regression lines with those of two different reference plasma samples.

Human Anti-Mouse Antibodies.
Anti-BT563 antibodies were detected in an ELISA system using microtiterplates precoated with BT563. Human antibodies were detected with an AP-conjugated goat antihuman-IgG antibody or goat antihuman-IgM (Jackson) and quantified by comparison with two different reference plasma samples. The concentration of anti-mouse-IgG antibodies in these reference plasmas was 54 and 154 mg/l respectively. For IgM antibodies an arbitrary Unit was defined.
The physicians on the Transplantation Department and the physician collecting all clinical data (TvG) were blinded for the results of peripheral blood monitoring, BT563 levels and antibody-levels.

Rejection-monitoring.
The diagnosis of acute rejection was made on clinical grounds by the responsible nephrologist, and was confirmed by core needle biopsy. Clinical indicators of acute rejection included rising creatinine, oliguria, fever and graft tenderness, in the absence of mechanical causes of renal dysfunction or toxic CsA-levels. Acute rejections were treated initially with 1 gram i.v. of methylprednisolone (Solu-Medrol, Upjohn) on three consecutive days. Steroid-resistant rejections were treated with rabbit-antithymocyte globuline (r-ATG). If an acute rejection occurred and anti-rejection therapy had to be started within the first ten post-operative days the study-treatment was continued.
For statistical analysis the two-tailed Fisher's Exact Test was used.

6.4 Results

BT563 tolerance.

The anti IL-2R antibody BT563 was tolerated remarkably well. Its use was not accompanied by the cytokine release syndrome, consisting of fever, hypotension and confusion, and treatment did not have to be discontinued in any patient for clinical intolerance. Only one BT563 treated patient experienced severe nausea with vomiting during six days, whereas in the placebo-group one patient temporarily complained of arthralgia, rash and low-grade fever.

Peripheral blood monitoring/ BT563 levels/ antiBT563 antibodies.

In the placebo group no changes in the proportions of CD3, CD4, CD8 and CD25-positive cell populations were observed. Within one day after the first injection of BT563, free binding sites on CD25 positive cells were no longer detectable in peripheral blood, using the MoAb 2A3, whereas CD3, CD4 and CD8 positive cells remained present in constant relative numbers. Staining with Rat-anti-Mouse antibody showed coating of all CD25 positive cells. Using labeled 2A3, no free binding sites on the IL-2R could be detected. After the last dose of BT563, CD25 remained coated with the antibody during a period of several weeks (median = 14 days; range = 3-26 days). CD25 always appeared at once on 20 - 25% of T cells, without an increase in the absolute number of CD3 positive cells. This is also indicative of coating of the CD25 molecule from activated T cells rather than elimination of cells from the circulation as seen during treatment with OKT3[14].
BT563 trough levels reached a plateau phase after day 4. The mean trough level at day 9 was 5.14 ± 0.40 μg/ml. The mean T½ for this monoclonal was 42 ± 5 hrs. A more than 4-fold increase from baseline in human anti-mouse antibodies (HAMAs) was found in 13/28 (46%) patients for IgM and in 4/28 (14%) patients for IgG. This increase in IgM-HAMAs was reached in the third and fourth week post-transplant in 85% of patients. In 5/13 the HAMA-response coincided with disappearance of BT563 from peripheral blod, in 6/13 patients this occurred at least 1 week after disappearance of BT563, wheras in 2/13 patients this occurred just before BT563-clearance.

Acute Tubulus Necrosis/ (Delayed) Graft Function.

In both groups a relatively small number of patients needed temporary renal

replacement therapy for delayed graft function (7/30 and 5/27 for placebo and BT563-treated patients respectively). The duration of dialysis requirement was not statistically different between the two groups (placebo: mean = 17 days (range = 4-31); BT563: mean = 10 days (range = 2-19)). At 1-year follow-up median serum creatinine levels were not significantly different (placebo: median = 141μmol/l (range = 74-328); BT563: median = 157μmol/l (range = 87-305)).

Rejection incidence.

In 7 patients in the placebo-group a rejection occurred during the first 10 post-operative days. Treatment with steroids resulted in reversal of this rejection episode in 4/7 patients. In the other 3 patients the rejection was steroid-resistant and a subsequent course of r-ATG had to be given, which was successful in only 1 patient. In the other 2 patients the rejection resulted in graft loss. In the BT563-group no rejection episodes were diagnosed during the prophylactic treatment period ($p = 0.011$; see Table 6.2). Within the first 4 post-operative weeks in the BT563-group only 1 steroid-responsive rejection occurred. In the subsequent 8 weeks 1 rejection episode occurred in the placebo-group and 2 occurred in the BT563-group. Thereafter a short course of methylprednisolon proved to be neccessary both in a BT563 and in a placebo-treated patient after conversion from cyclosporin to azathioprine medication at 6 months after transplantation. Actual freedom from rejection at one year in the patients at risk was 85 % in the BT563-group and 68% in the placebo-group.

Table 6.2
Patients with acute rejection after kidney transplantation

Days after tx	Placebo	BT563	p-value*
0 - 10	7/29	0/27	$p = 0.011$
0 - 28	7/29	1/27	$p = 0.052$
0 - 90	8/29	3/27	$p = 0.181$
0 -365	9/28	4/27	$p = 0.205$

Infectious complications.

Table 6.3 shows the infectious complications. No significant differences between the two groups were found. In 2 placebo-treated patients CMV-disease followed rejection treatment with steroids and/or r-ATG. One BT563 treated patient experiencing a primary EBV-infection underwent tracheostomy because of a large pharyngeal lymphomatous swelling, which disappeared after tapering of the CsA dosage[15].

Table 6.3 Infectious complications after kidney transplantation.

	week 0 - 4		week 5 - 12	
Type of infection	placebo n = 29	BT563 n = 27	placebo n = 28	BT563 n = 27
Bacterial				
urinary tract infection	8	8	4	8
pneumonia	1	0	0	0
perirenal infection	0	1	0	1
endocarditis lenta	0	1	0	0
S. aureus sepsis	0	0	1	1
Viral				
CMV - disease	0	0	3	0
herpes genitalis	0	0	1	0
herpes zoster	0	0	0	1
primary EBV - infection	0	0	0	1
Fungal				
Candida albicans stomatitis	0	1	0	0
Candida albicans peritonitis	1	0	0	0

Graft and patient survival.

Two grafts were lost within the first 5 postoperative days due to technical failure (difficult vascular anastomosis and torsion of vessels) and another graft was lost due to renal artery thrombosis (all three in the BT563 group). At 6 months and at 11 months two patients in th BT563 group lost their grafts due to chronic rejection. In the placebo group two patients lost their graft due to acute rejection, one of them died three months later from uremia and sepsis. One placebo-treated patient suffered from a non-fatal myocardial infarction on the second post-operative day; 4 months later he was resuscitated outside the hospital and died a few days later. Acute necrotizing pancreatitis developed in another placebo-treated patient at day 4; this patient was removed from the study and died at day 32. Actual overall one year graft survival was 84% for the BT563 patients and 87% for the placebo-group. Patient survival at 1 year was 100% in the BT563 and 90% in the placebo group.

Other complications.

A spontaneously resolving ileus lasting 1 week occurred in a placebo-treated patient immediately after transplantation. One BT563 treated patient was found to have Menetrier's disease two months after transplantation. Two other patients in the BT563 group had a squamous cell carcinoma (one localized in the pharynx, detected three months after transplantation, and one on the skin of the lower leg, diagnosed eight months after transplantation). Both tumors were treated radically.

6.5 Discussion.

This randomized prospective trial is the first to investigate the efficacy and safety of an anti IL-2R MoAb in the prevention of kidney allograft rejection in a double-blind fashion. It shows a beneficial effect of BT563 in the prevention of early rejection after kidney transplantation. At 12 weeks the difference between the two groups is 3 vs. 8 patients with a rejection (Figure 6.1) and 2 graft losses due to rejection in the placebo-group. At 1-year follow-up the overall graft and patient

survival in the BT563 and placebo group were 84% vs 87% and 100% and 90%. As is shown in Table 6.3 the applied use of BT563 did not result in a higher incidence of infectious complications. CMV infections were more frequent in the placebo-group, probably due to the required use of rejection-treatment in this group. In contrast to the infectious complications there was an unfortunate occurrence of two squamous cell carcinomas in the BT563-group, but these were

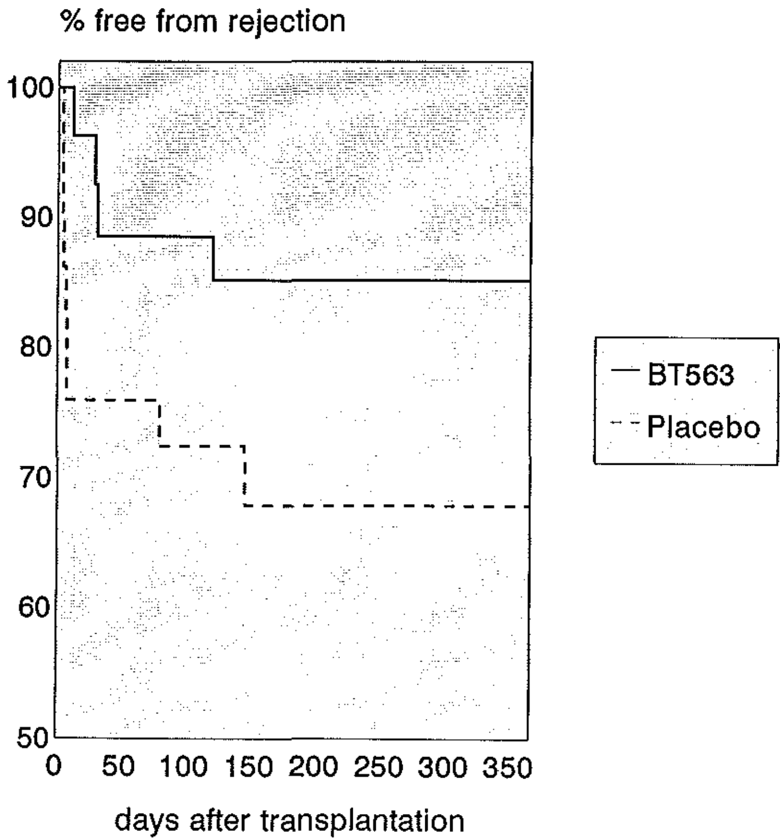

Figure 6.1. Freedom from acute rejection after kidney transplantation.
(- - - - = Placebo ______ = BT563)

treated curatively and did not lead to death. Drug related side effects were not observed. Unexpected was the occurrence of renal artery thrombosis in one BT563 treated patient. Vascular thrombosis is a well known complication of the use of mono- and polyclonal anti pan-T-cell preparations[16 17]. It is unclear whether this is also the case for anti IL-2R MoAbs, for which this has not been reported before.

In 1990 Soulillou et al published the results of a study[6] comparing 33B3.1 (another anti IL-2R MoAb) with r-ATG for rejection prophylaxis in kidney transplantation. In this study a rejection occurred in 6/49 patients (12%) during the 14-day course of 33B3.1, whereas in our study no BT563 treated patient had a rejection during the prophylactic treatment. In our study during the treatment-phase CD25+ cells in peripheral blood were coated, and free binding sites on the IL-2R returned only after a median of 14 days after this 10-day course. Soulillou described that uncoated CD25+ cells returned during the 33B3.1 course. The most likely explanation for this difference is the fact that in the latter study cyclosporin was started on day 14 (at the end of the MoAb-administration), whereas in our study cyclosporin was started on day 0. Cyclosporin inhibits the expression of IL-2 at the transcriptional level[18], but only partially inhibits the synthesis of the IL-2R[19]. In animal studies a synergistic effect of combining cyclosporin and anti IL-2R MoAbs has been demonstrated, which probably is the result of influencing different steps in the immune cascade[20 21].
In the study by Kirkman et al[7] rejection episodes also occurred during treatment with the anti IL-2R MoAb anti-Tac (5/40). Cyclosporin in this study was started on day 0. In contrast to the 33B3.1 and our study, uncoated CD25+ cells remained present in peripheral blood throughout treatment, reflecting the limited potency of anti-Tac MoAb. The differences in efficacy between several anti IL-2R MoAbs may be due to affinity and binding of the MoAbs to different epitopes of the IL-2R.

In conclusion the use of BT563 in combination with cyclosporin is effective in preventing acute rejection after kidney transplantation without being associated with a higher incidence of infectious complications. In view of the two squamous cell carcinomas in the BT563 group this must be carefully followed in future trials.

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Chapter 7

Reversal of heart allograft rejection with monoclonal anti-interleukin-2 receptor antibody.

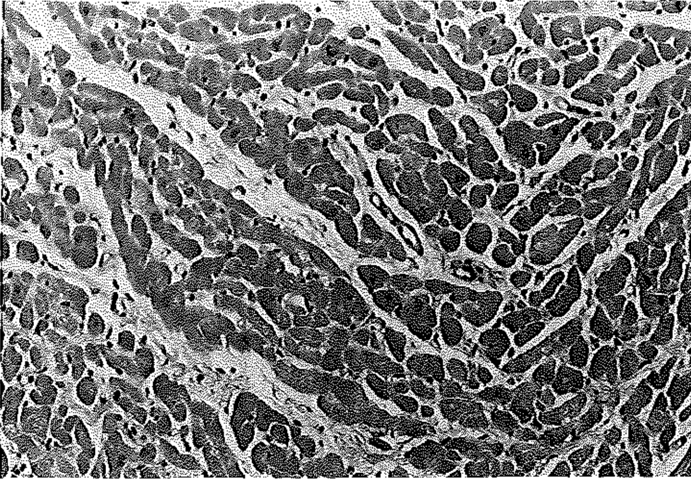
This chapter has been published in abstract form as a Letter, entitled "Reversal of graft rejection with monoclonal anti-interleukin-2 receptor", in The Lancet 1992;339:873 by Gelder van T, Balk AHMM, Mochtar B, Weimar W.

7.1 Introduction

Since 1987 monoclonal antibodies (MoAb) to the interleukin-2 receptor (IL-2R) have been used in clinical organ transplantation[1]. Although some preparations were shown to be effective in the prevention of rejection after kidney[2] and liver[3] transplantation, their efficacy in rejection treatment is still speculative[4]. In heart transplantation in animals, anti IL-2R MoAbs were effective both in prevention[5] and treatment[6] of rejection. We report succesful treatment with BT563, a murine IgG_1 anti IL-2R MoAb, in three heart transplant recipients with acute graft rejection.

7.2 Patients and methods

Two men and one woman (ages 57, 58 and 59 years respectively) underwent heart transplantation in the first half of 1991 because of end-stage heart failure due to dilated cardiomyopathy. Maintenance immunosuppression consisted of cyclosporin and prednisone. After transplantation they experienced several rejection episodes, for which they received pulsed doses of methylprednisolone, OKT3 and rabbit antithymocyte globulin (RIVM, Bilthoven, The Netherlands). An overview of the clinical characteristics of these three patients is given in Table 7.1. All three patients had a recurrence of rejection graded 3A (Figure 7.1), according to the criteria of the International Society for Heart Transplantation[7]. The episodes under study (fourth or fifth rejection episode respectively) were treated with BT563 (10 mg intravenously for 10 days in one patient; 5 mg iv for 10 days in the two others).



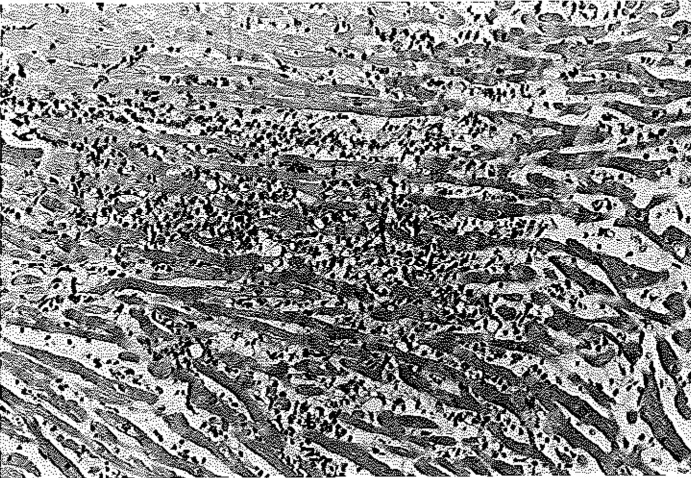


Figure 7.1 Microscopy of heart allograft endomyocardial biopsies, showing (A) no rejection (grade 0), and (B) multifocal aggressive infiltrates with myocyte damage (grade 3A).

Table 7.1 Clinical characteristics of 3 heart allograft recipients.

	patient 1	patient 2	patient 3
age	57	59	58
sex	M	F	M
heart disease	dilated CMP	dilated CMP	dilated CMP
panel reactive antibodies	4%	96%	0%
date of transplant	18.03.1991	15.06.1991	28.05.1991
HLA mismatches A/B/Dr	2/2/1	2/1/2	1/1/1
current rejection date	06.01.1992	31.10.1991	17.12.1991
grade	3A	3A	3A
rejection episode	5th	4th	5th
daily dose BT563	5 mg	10 mg	5 mg

7.3 Results

Treatment with BT563 in these three heart transplant recipients did not result in any clinically significant side-effect. After the first dose of BT563 all CD25 positive cells in peripheral blood were coated with the anitbody, in both patients treated with a daily dose of 5 mg as well as in the patient treated with 10mg daily. Trough levels reached 3500 ng/ml in the 5 mg treated patients and 9500 ng/ml in the patient receiving 10 mg daily. Twelve days after the last dose of BT563 a control endomyocardial biopsy was taken from all three patients, showing grade 1A in one patient and grade 0 in the other two.

7.4 Discussion

Analysis of the cellular infiltrate present in rejecting cardiac allografts has shown an accummulation of large numbers of donor-specific cytotoxic T-lymphocytes, correlated with extensive inflammatory endothelial development and myocyte

destruction[8]. Interleukin-2 produced by these CD4 positive T-cells directly promotes their proliferation and promotes the production of other cytokines like interferon-γ, contributing to the development of inflammation. IL-2R directed therapy may interrupt the rejection cascade.

Early experience with BT563 in the treatment of rejection was obtained in the succesful treatment of acute graft-versus-host disease (GVHD), refractory to corticosteroids[9].

The three patients described in this paper had already been treated with OKT3, ATG and Solu-Medrol for previous rejection-episodes. During and after treatment of rejection sensitization to OKT3 and ATG may have occurred, potentially leading to serum sickness when retreated with these agents. Even worse, sensitization to OKT3 has been related to the development of vascular rejection in cardiac allograft recipients leading to increased graft loss[10]. Therefore the decision was made to treat this rejection episode with BT563. The administration of BT563, similar to patients receiving this drug for rejection-prophylaxis, did not result in any adverse side-effect and monitoring of peripheral blood showed coating of CD25 positive cells.

Control endomyocardial biopsies in all 3 patients showed resolved rejection (biopsy grades 0 or 1A). We therefore conclude that BT563 is a safe and efficacious drug in the treatment of acute rejection in heart allograft recipients.

7.5 References

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Chapter 8

Summary and conclusions.

8.1 Summary and conclusions.

Monoclonal and polyclonal antibody preparations have been used for induction therapy after organ transplantation. Apart from serious side effects directly associated with their use, e.g. the cytokine release syndrome, the resulting deep and aspecific immunosuppression leads to increased incidences of infections and in the long term of malignancies. To avoid the drawbacks of this aselective immunosuppression monoclonal antibodies against specific T-cell activation determinants have been developed. In view of the pivotal role of Interleukin-2 in the activation cascade of antigen-stimulated lymphocytes, monoclonals directed against the Interleukin-2Receptor were thought to have a strong inhibitory effect on activated T-lymphocytes only.

We have performed in vitro studies using several different anti IL-2R monoclonals because not all anti IL-2R mAb are equally effective. Depending on the epitope recognized, the affinity of the mAb, type of F_c-receptor, the resulting human anti-mouse antibody response and the T½ of the mAb, the in vivo efficacy may vary. For some mAbs the results of in vitro experiments seem to be reflected in immunosuppressive potential. In vitro experiments with anti-Tac, in comparison with BT563 and 33B3.1, showed only limited potency of anti-Tac in inhibiting mixed lymphocyte reactions. BT563 was able to strongly inhibit lymphocyte activation in concentrations that can easily be reached in a clinical setting. A pilot study in 12 renal transplant recipients showed excellent tolerance, serum levels that in vitro were efficacious and coating with the antibody of all CD25 positive cells in peripheral blood during prophylactic BT563 treatment. None of these 12 patients showed signs of rejection.

This made BT563 an attractive and promising drug and in 1991 we started two large clinical trials with BT563 as a prophylaxis against rejection after solid organ transplantation.

Sixty first and second renal transplant recipients were randomized for double-blind prophylactic treatment with either placebo or BT563 during the first 10 days after transplantation. From day 0 all patients received prednisone and cyclosporin as maintenance treatment. Drug related side effects were not observed. During the 10-day course of BT563 no rejections were found, whereas during placebo-treatment in 7/29 patients an acute rejection occurred ($p=0.01$). At 4 weeks after

transplantation freedom from rejection in the BT563 and placebo-group were 96% and 76% respectively (p=0.05). Due to rejection in the placebo-group 2 grafts were lost. Infectious complications were equally distributed between the two groups. It was concluded that BT563 effectively prevents rejection after kidney transplantation and does not lead to drug-related side-effects or to an increased incidence of infections.

The other study was performed in 60 heart transplant recipients, who were randomized for a 7-day course of either BT563 or OKT3 as rejection-prophylaxis. These patients also had prednisone and cyclosporin as maintenance treatment, but cyclosporin here was started on day 3. In the first week the majority (27/29) of OKT3 treated patients suffered from one or more symptoms of the cytokine release syndrome (fever, hypotension, psychosyndrome, rash). In some (13/31) of the BT563 treated patients low-grade fever and mild confusion were found, but these were not life-threatening and are often seen following open heart surgery. First acute rejections in the BT563 group were often found in the first or second week (14/29=48%), whereas first acute rejections in the OKT3 group usually occurred a little later (in weeks 3, 4 or 5: 15/29=52%). However, at 3 months after transplantation freedom from acute rejection between the two groups was not significantly different (17 vs 21%). Neither was there a difference in the amount of steroids or anti T-cell treatments (OKT3/ATG) used for rejection-treatment. Infectious complications were equally distributed in both groups. The early rejections in the BT563 group at week 1 occurred after one week of treatment, in the presence of seemingly adequate concentrations of BT563 in the circulation and in a situation of complete CD25-coating in peripheral blood. To see whether uncoated CD25-positive cells could be found within the rejecting grafts immunohistologic studies on the week 1-biopsies were done. In 80% of the rejecting patients uncoated CD25-positive cells were present within the endomyocardial biopsies, whereas these cells could be detected in only 22% of non-rejecting grafts. RT-PCR studies on a limited number of biopsies showed the presence of mRNA for IL-2 in 3/5 (60%) of biopsies of rejecting grafts. An explanation for finding uncoated CD25-positive cells in the presence of effective BT563 plasma levels may be found in the low distribution volume of the monoclonal, leading to insufficient tissue concentrations.

An important difference between the renal and heart transplant study is the fact

that renal transplant recipients started using cyclosporin on day 0, whereas in heart transplant recipients this was delayed until day 3, in order to prevent renal insufficiency in the immediate postoperative period. In animal studies a synergistic effect of combining cyclosporin and anti IL-2R mAbs has been demonstrated. Possibly the results of rejection-prophylaxis with BT563 in the heart transplant recipients would have been different if cyclosporin had been started on day 0, thereby making optimal use of the synergistic effect of combining BT563 and cyclosporin.

An overview of all published randomized trials using anti IL-2R mAbs in organ transplantation is presented on page 105. In conclusion, the use of anti IL-2R MoAbs as a prophylaxis against rejection after organ transplantation is safe and does not lead to an increased incidence of infectious complications. Their efficacy in kidney transplantation is now shown by several studies[1 2 3 5]. In liver transplantation considerable differences between the results obtained with several different anti IL-2R mAbs have been found[7 8]. BT563 as a prophylaxis against rejection in liver transplantation was most effective[9 10]. In our study using anti IL-2R mAb in human cardiac transplantation BT563 seems equally effective in preventing acute rejection compared to OKT3, although rejections tend to come a little sooner[11]. Combining anti IL-2R MoAbs with cyclosporin from the day of transplantation is most effective, as shown by studies in liver transplantation[8 9 10] and by our kidney study[5].

Although the observed efficacy of BT563 in the treatment of rejection in three heart transplant recipients is promising, it can not be concluded that anti IL-2R monoclonals are effective in the treatment of established graft rejection. In solid organ transplantation experience with anti IL-2R monoclonals for this indication is limited. Results of treating graft-versus-host disease after bone marrow transplantation are inconclusive.

Whether humanized antibodies, that are markedly less immunogenic and have improved pharmacokinetics with prolonged in vivo survival, have equal or even better efficacy and safety remains to be proven. The long half life may potentially lead to prolonged periods of CD25-absence and over-immunosuppression.

RANDOMIZED TRIALS OF ANTI IL-2R INDUCTION TREATMENT IN ORGAN TRANSPLANTATION

Organ	Year of Publication (Ref.)	MoAb	Control Group	Duration of Treatment	Combined with	OUTCOME		
						Rejection Incidence	Infections	Other side Effects
kidney	1990 (1)	33B3.1	rATG	14	pred/aza later CsA	33B3.1=rATG	33B3.1<rATG	33B3.1<rATG
	1991 (2)	antiTac	-	10	triple	antiTac<control	antiTac=control	antiTac=control
	1993 (3)	Lo-Tact-1	ALG	14	triple	Lo-Tact-1=ALG	Lo-Tact-1<ALG	Lo-Tact-1=ALG
	1994 (4)	33B3.1	rATG	10	pred/aza later CsA	33B3.1>rATG	33B3.1<rATG	33B3.1=rATG
	1995 (5)	BT563	placebo	10	CsA/pred	BT563<placebo	BT563=placebo	BT563=placebo
kidney pancreas	1994 (6)	33B3.1	rATG	10	triple	33B3.1>rATG	33B3.1=rATG	33B3.1=rATG
liver	1991 (7)	YTH-906	placebo	10	triple	YTH-906=placebo	YTH-906=placebo	YTH-906=placebo
	1993 (8)	Lo-Tact-1	OKT3	10	triple	Lo-Tact-1=OKT3	Lo-Tact-1<OKT3	Lo-Tact-1=OKT3
		Lo-Tact-1	placebo	10	triple	Lo-Tact-1<placebo	Lo-Tact-1=placebo	Lo-Tact-1=placebo
	1993 (9)	BT563	ATG	10	triple	BT563<ATG	BT563=ATG	BT563=ATG
	1995 (10)	BT563	ATG	12	triple	BT563<ATG	BT563=ATG	BT563<ATG
heart	1996 (11)	BT563	OKT3	7	CsA/pred	BT563=OKT3	BT563=OKT3	BT563<OKT3

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Chapter 9

Samenvatting en conclusies.

9.1 Samenvatting en conclusies

Diverse monoclonale en polyclonale antilichamen werden in de afgelopen jaren gebruikt als inductie therapie na orgaan transplantatie. Naast de directe bijwerkingen gepaard gaande met hun gebruik, zoals het "cytokine release syndrome", resulteert de diepe immunosuppressie veroorzaakt door deze middelen in een toegenomen incidentie van infecties en op den duur van maligniteiten. Ter voorkoming van de nadelen die kleven aan het gebruik van dergelijke aspecifiek werkende immunosuppressiva werden monoclonale antilichamen ontwikkeld die gericht zijn tegen specifieke T-cel activatie antigenen. Gezien de centrale rol van Interleukine-2 (IL-2) bij de activatie van antigen-gestimuleerde lymfocyten leken antilichamen gericht tegen de Interleukine-2 receptor (IL-2R) een goed voorbeeld van een krachtig immunosuppressief middel gericht tegen alleen geactiveerde lymfocyten.
In vitro studies met verschillende anti IL-2R monoclonalen lieten zien dat er onderlinge verschillen zijn in effectiviteit. Afhankelijk van de epitoop die wordt herkend, de affiniteit van het monoclonaal, het type F_c-receptor, de resulterende anti-muis antilichaam respons en de halfwaardetijd van het antilichaam varieert de in vivo werkzaamheid. Voor sommige monoclonalen geldt dat de effectiviteit in vivo lijkt te worden weerspiegeld in de resultaten van in vitro experimenten. Zo blijkt de immunosuppressieve invloed van anti-Tac in vitro, in vergelijking met die van 33B3.1 en BT563, beperkt te zijn. BT563 bleek in concentraties die klinisch goed haalbaar zijn een sterke remming te geven van de proliferatie in mixed lymphocyte reactions. BT563 leek dan ook een goede keuze te zijn om in vivo studies bij niertransplantatie patienten te starten.
Een pilot studie, waarin aan 12 niertransplantatie patienten BT563 als inductietherapie werd toegediend, toonde dat in perifeer bloed alle CD25 positieve cellen tijdens de behandeling afgedekt worden met het antilichaam en dat het middel geen bijwerkingen veroorzaakte. Bovendien bleek geen van alle 12 patienten na transplantatie een rejectie te krijgen.
De veelbelovende resultaten van het in vitro werk en van de pilot studie waren aanleiding om in 1991 twee grote gerandomizeerde klinische studies te starten, waarbij BT563 werd toegepast ter preventie van rejectie na nier- en harttransplantatie.

Zestig eerste en tweede niertransplantaat ontvangers werden gerandomizeerd voor dubbel-blinde behandeling met placebo of BT563 gedurende de eerste 10 dagen na transplantatie. Vanaf dag 0 ontvingen alle patienten prednison and cyclosporine als onderhoudsmedicatie. Aan de medicatie gerelateerde bijwerkingen werden niet waargenomen. Tijdens de behandeling met BT563 kreeg geen enkele patient een afstoting, terwijl in de placebo-groep tijdens de eerste 10 dagen 7/29 patienten een acute rejectie hadden ($p = 0.01$). Vier weken na transplantatie was 96% resp. 76% van de patienten vrij van afstoting in de BT563 en placebo-groep ($p = 0.05$). Als gevolg van rejectie gingen in de placebo-groep twee transplantaten verloren. Infecties waren gelijkelijk verdeeld over beide groepen. De conclusie van dit onderzoek luidt dat BT563 effectief is in het voorkomen van afstoting na niertransplantatie, zonder dat dit leidt tot vervelende bijwerkingen of een toegenomen frequentie van infecties.
Een tweede onderzoek werd verricht onder 60 harttransplantatie patienten, die werden gerandomizeerd voor behandeling met BT563 of OKT3 als rejectie-profylaxe. Deze patienten hadden ook prednison en cyclosporine als onderhoudsmedicatie, maar cyclosporine werd pas gestart op dag 3 na transplantatie. De meeste (27/29) met OKT3 behandelde patienten ondervonden één of meer verschijnselen van het "cytokine release syndrome" (koorts, hypotensie, verwardheid, rash). Een aantal (13/31) van de met BT563 behandelden had enige verwardheid of subfebriele temperatuur in de direct post-operatieve fase, maar dit bleek niet levensbedreigend en was niet anders dan wat ook normaliter na open-hart chirurgie gezien wordt. Acute rejectie werd in de BT563-groep vaak gezien in het eerste biopt (week 1) na transplantatie (14/29 = 48%), terwijl dit in de met OKT3 behandelde patienten veelal werd gevonden in de biopten genomen op week 3, 4 of 5 (15/29 = 52%). Drie maanden na transplantatie was het verschil in afwezigheid van afstoting tussen de twee groepen niet meer aanwezig (17 vs 21%). Evenmin was er een verschil tussen de twee groepen in de vanwege afstoting toegediende hoeveelheden corticosteroiden of in de aantallen kuren anti T-cel therapie (OKT3/ATG). Ook hier waren ernstige en minder ernstige infecties in beide groepen even frequent.
In de harttransplantatie studie werd dus bij enkele patienten een afstoting in het transplantaat geconstateerd terwijl zij net de BT563 behandeling hadden ontvangen en er bij hen in perifeer bloed alleen door het monoclonaal afgedekte CD25

positieve cellen in perifeer bloed aantoonbaar waren. Om te onderzoeken of wellicht in het hart wel vrije IL-2 bindingsplaatsen (onbedekt CD25) aanwezig waren werd immunohistochemisch onderzoek verricht op de week 1 biopten. In de endomyocardbiopten van 80% van de rejecterende patienten bleken onbedekte CD25 positieve cellen aantoonbaar te zijn, terwijl dit bij de niet rejecterende patienten slechts in 22% van de patienten het geval was. RT-PCR onderzoek op een beperkt aantal biopten toonde bovendien aanwezigheid van mRNA voor IL-2 in 3/5 (60%) van de rejectie-biopten. Een verklaring voor het feit dat BT563 blijkbaar wel in de circulatie maar niet in het transplantaat zelf in staat was CD25 volledig af te dekken zou gelegen kunnen zijn in het lage verdelingsvolume van het monoclonaal.
De twee onderzoeken vergelijkend lijkt het zo te zijn dat BT563 bij de niertransplantatie patienten wel en bij de harttransplantatie patienten minder effectief is. Een belangrijk verschil tussen de twee onderzoeken is dat in de nieren-studie gestart werd met cyclosporine op dag 0, terwijl dit bij de harttransplantaties werd uitgesteld tot dag 3, ter voorkoming van ernstige nierfunctiestoornissen in de direct post-operatieve periode. In dierexperimenteel onderzoek werd een duidelijk synergistisch effect gevonden bij het combineren van cyclosporine en anti IL-2R monoclonalen. Mogelijk zouden de resultaten bij de harten-studie anders zijn geweest als daar ook was gestart met cyclosporine op de eerste post-operatieve dag.

Een overzicht van alle tot heden gepubliceerde gerandomizeerde onderzoeken met anti IL-2R monoclonale antilichamen als inductie therapie na orgaantransplantatie wordt gegeven op pagina 114. Concluderend kan worden gesteld dat het gebruik van anti IL-2R monoclonalen na orgaan transplantatie veilig is en niet leidt tot een toename van infecties. De effectiviteit van IL-2R monoclonalen ter preventie van rejectie na niertransplantatie, zoals ook gevonden door anderen[1 2 3], kan door ons[5] worden bevestigd. In de literatuur worden na levertransplantatie wisselende resultaten gemeld[7 8], maar met name BT563 lijkt hierbij het meest effectief[9 10]. In ons onderzoek met BT563 na harttransplantatie blijkt dat dit gelijkwaardig is aan OKT3, ofschoon de rejecties wel wat eerder na transplantatie optreden[11]. Op grond van dierexperimenteel onderzoek, onze resultaten bij de niertransplantatie patienten en de beschreven effecten na levertransplantatie wordt geadviseerd BT563 te

combineren met cyclosporine.
Ofschoon de beschreven effectiviteit van BT563 als behandeling van een aangetoonde rejectie na harttransplantatie veelbelovend is, kan niet worden geconcludeerd dat anti IL-2R monoclonalen bij allograft rejectie werkzaam zijn. Hiernaar is nog onvoldoende onderzoek verricht.
Of gehumaniseerde anti IL-2R monoclonalen, die minder immunogeen zijn en langere halfwaardetijden hebben, dezelfde of misschien zelfs betere effectiviteit hebben valt te bezien. Met name de lange halfwaardetijd zou kunnen leiden tot overimmunosuppressie met alle gevolgen van dien.

RANDOMIZED TRIALS OF ANTI IL-2R INDUCTION TREATMENT IN ORGAN TRANSPLANTATION

Organ	Year of Publication (Ref.)	MoAb	Control Group	Duration of Treatment	Combined with	OUTCOME		
						Rejection Incidence	Infections	Other side Effects
kidney	1990 (1)	33B3.1	rATG	14	pred/aza later CsA	33B3.1=rATG	33B3.1<rATG	33B3.1<rATG
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	1993 (3)	Lo-Tact-1	ALG	14	triple	Lo-Tact-1=ALG	Lo-Tact-1<ALG	Lo-Tact-1=ALG
	1994 (4)	33B3.1	rATG	10	pred/aza later CsA	33B3.1>rATG	33B3.1<rATG	33B3.1=rATG
	1995 (5)	BT563	placebo	10	CsA/pred	BT563<placebo	BT563=placebo	BT563=placebo
kidney pancreas	1994 (6)	33B3.1	rATG	10	triple	33B3.1>rATG	33B3.1=rATG	33B3.1=rATG
liver	1991 (7)	YTH-906	placebo	10	triple	YTH-906=placebo	YTH-906=placebo	YTH-906=placebo
	1993 (8)	Lo-Tact-1	OKT3	10	triple	Lo-Tact-1=OKT3	Lo-Tact-1<OKT3	Lo-Tact-1=OKT3
		Lo-Tact-1	placebo	10	triple	Lo-Tact-1<placebo	Lo-Tact-1=placebo	Lo-Tact-1=placebo
	1993 (9)	BT563	ATG	10	triple	BT563<ATG	BT563=ATG	BT563=ATG
	1995 (10)	BT563	ATG	12	triple	BT563<ATG	BT563=ATG	BT563<ATG
heart	1996 (11)	BT563	OKT3	7	CsA/pred	BT563=OKT3	BT563=OKT3	BT563<OKT3

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Curriculum vitae.

De auteur van dit proefschrift werd geboren op 16 september 1962 in Vlaardingen. Na het behalen van het VWO-diploma aan de Scholengemeenschap Hugo Grotius te Delft startte hij in 1980 met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Hier werd in februari 1987 het artsexamen verkregen, waarna de opgedane medische kennis in praktijk werd gebracht tijdens de militaire dienstplicht die werd vervuld op het Artillerie Schietkamp in Oldebroek. Vervolgens werd in mei 1988 begonnen aan de opleiding tot internist in het Dijkzigt Ziekenhuis in Rotterdam (opleider: Prof.Dr.M.A.D.H. Schalekamp). Na het voltooien van de opleiding tot internist werd het aandachtsgebied nefrologie (opleider: Prof.Dr.W. Weimar) bestudeerd, leidend tot registratie als internist-nefroloog per 1 december 1995. Gedurende de opleiding tot internist werd gestart met het in dit proefschrift beschreven onderzoek. Voor een presentatie over dit onderzoek werd hij onderscheiden met de Nefrologie Prijs 1994 van de Nederlandse Vereniging voor Nefrologie. Sinds november 1995 is hij als staflid werkzaam op de afdeling Interne Geneeskunde I. Op 4 april 1996 ontving hij de tweejaarlijkse "Jon J. van Rood-prijs", vanwege zijn wetenschappelijk werk op het gebied van de transplantatiegeneeskunde.

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endomyocardbiopten heeft beoordeeld, en van Dries Mulder voor zijn bijdrage aan hoofdstuk 5, wordt zeer gewaardeerd.
Omdat er behalve goede klinische zorg ook nog eens een wetenschappelijk hoogwaardig transplantatie-laboratorium in dit ziekenhuis is kon aan het onderzoek de nodige diepgang worden geboden. De medewerkers van dit laboratorium hebben vele duizenden buisjes bloed en talloze hartbiopten verwerkt. Ik ben met name ook hen veel dank verschuldigd en wil Nicolette Jutte, Cees Hesse, Len Vaessen, Carla Baan, Nicole van Besouw, Nancy van Emmerik, Rene Daane, Christiaan Knoop, Lisette Loonen en Wendy Mol hier noemen. Ik hoop ook in de komende jaren nog veel (en vaker) op het lab te zijn en de transplantatie-puzzle verder te completeren.
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