Anti-IL-2Rα therapy in renal transplantation: The promise of steroid-free immunosuppression?

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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Chapter 1

Introduction and outline of the thesis

In the last two decades the results of renal transplantation have tremendously improved (1). With the availability of new and potent immunosuppressive drugs, the incidence of acute rejection has decreased to less than 25%; graft survival at one year now ranges from 80 to 100% (2;3). However, recipients of a renal transplant frequently experience several side effects related to the use of the immunosuppressive drugs. Well known adverse effects are an increase in cardiovascular risk factors (4) with ensuing cardiovascular morbidity (5), an increased susceptibility to infections (6;7) and malignancies (8), an increased bone loss leading to bone fractures (9), and a progressive loss of renal function (10). In our endeavor to develop optimal immunosuppressive treatment protocols we therefore must weigh the benefits of immunosuppression (less rejection episodes, improved graft survival) against the side effects. Recently, immunosuppressive agents with a more specific mode of action have become available, that offer the prospect of more effective immunosuppression and fewer side effects. Examples are basiliximab and daclizumab, monoclonal antibodies against alpha chain of the interleukin-2 receptor (IL-2Rα), respectively.

In this thesis we describe our experience with daclizumab. Specifically, we questioned if the use of daclizumab would allow steroid-free immunosuppression.

In *chapter two*, the usage of monoclonal antibodies against IL-2Rα in renal transplantation is reviewed. In the clinical studies that have been done with daclizumab, the treatment protocols required the administration of five i.v. bolus injections of daclizumab at regular intervals after transplantation (11;12). However, on theoretical grounds, daclizumab should also be effective when used in a lower dose. Therefore we decided to limit the use of daclizumab to two i.v. bolus injections administered immediately before and two weeks after transplantation. We have evaluated the duration of blockade of the IL-2Rα on peripheral lymphocytes with this dosing schedule *(chapter three)*. Measurement of IL-2Rα on peripheral lymphocytes is tedious and time-consuming. Therefore, we have looked for an alternative method for monitoring of blockade of the IL-2Rα after treatment with daclizumab. Specifically, we have studied the urinary excretion of the soluble IL-2Rα during treatment with daclizumab *(chapter four)*. Not unexpectedly, we noted that daclizumab decreased the urinary excretion of soluble IL-2Rα, probably by binding soluble IL-2Rα, thus forming large complexes that cannot pass the glomerular barrier. In *chapter five* we present data to answer the question if measurement of urinary soluble IL-2Rα reflects the blockade of IL-2Rα on peripheral lymphocytes after treatment with daclizumab.

In the following two chapters we describe the efficacy of daclizumab as a substitute for the use of corticosteroids after renal transplantation. In our prospective, multicenter study renal transplant recipients were randomized for treatment with either daclizumab (two i.v. bolus injections at day 0 and 14) or corticosteroids for four months added to the standard immunosuppression with tacrolimus and mycophenolate mofetil. The results of this study are described in *chapter six* (overall results of graft and patient survival, rejection episodes, and cardiovascular risk factors) and in *chapter seven* (specific data on bone mineral density). Quantitative ultrasound of bone has been shown to measure the density as well as the microarchitecture of bone (13). In a subgroup of patients we have evaluated changes in quantitative ultrasound parameters, and compared the results obtained by this technique with data on bone mineral density as assessed by dual energy X-ray absorptiometry *(chapter eight)*.

Outline of the thesis

The studies described in this thesis provide answers to the following questions:

1. What is the duration of IL-2Rα blockade after administration of two doses of daclizumab? *(Chapter 3)*
2. What is the influence of daclizumab on the metabolism of soluble IL-2Rα? *(Chapter 4)*
3. Is it possible to predict blockade of IL-2Rα on peripheral lymphocytes after treatment with daclizumab by measuring the concentration of soluble IL-2Rα in serum and urine? *(Chapter 5)*
4. Is steroid-free renal transplantation feasible by using daclizumab in addition to an immunosuppressive regimen with tacrolimus and MMF? *(Chapter 6)*
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8. Are the changes in bone mineral density as assessed by dual energy X-ray paralleled by changes in quantitative ultrasound parameters during the first year after renal transplantation? *(Chapter 8)*

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Chapter 2

Monoclonal antibodies against the IL-2Rα: Highly effective immunosuppression without apparent side effects

Adapted from:

Daclizumab en basiliximab: Effectieve immuunsuppressie zonder bijwerkingen

CG ter Meulen, LB Hilbrands, IC van Riemsdijk, RJ Hené, MHL Christiaans, AJ Hoitsma.

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The results of solid organ transplantation have tremendously improved in the past two decades (1). Twenty years ago, when standard immunosuppression consisted of azathioprine and prednisone, only 60-65% of the patients had a functioning renal graft at one year after transplantation. Acute rejection episodes, occurring in up to 75% of the patients, were an important cause of early graft loss. With the use of new and potent immunosuppressive drugs, such as the calcineurin inhibitors cyclosporine or tacrolimus, or the antiproliferative drug mycophenolate mofetil, the incidence of acute rejection has decreased to less than 25%, and graft survival at one year now ranges from 80 to 100% (2;3).
The T- lymphocyte plays a pivotal role in the process of acute rejection. Therefore, anti T-cell antibodies were developed for the prevention, and treatment of acute rejection episodes. Polyclonal antibodies against the T-lymphocyte (e.g. antithymocyte globulin (ATG)) were generated by immunization of animals (rabbit, horse) with human T-lymphocytes. With the use of hybridomas, monoclonal antibodies directed at specific epitopes on the T-lymphocyte such as the CD3/T-cell receptor complex (e.g. OKT3) were produced. Both the polyclonal and monoclonal antibodies have proved effective for the treatment of acute rejection, however at the cost of an increased incidence of opportunistic infections and lymphoproliferative diseases (4). Besides, the first administration of these antibodies is frequently complicated by a massive release of cytokines, as a result of activation and lysis of T-lymphocytes, leading to high fever, arthralgias and capillary leakage. These side effects have limited the use of these antibodies, which are reserved for patients at high risk for acute rejection, or for treatment of steroid-resistant acute rejection episodes.

Daclizumab and basiliximab

The balance between immunosuppressive efficacy and adverse events is an important clinical problem. Daclizumab and basiliximab, monoclonal antibodies directed against the alpha chain of the interleukin 2 receptor (IL-2Rα), seem an important advance by providing highly selective immunosuppression without major adverse events. In this article the mechanism of action, the pharmacokinetic and -dynamic data, and the results of clinical studies with these monoclonal antibodies in renal transplantation are discussed. Finally, we discuss the indication for using these monoclonal antibodies, in the perspective of the currently available immunosuppressive drugs.

Mechanism of action

The interaction between interleukin-2 (IL-2) and the interleukin-2 receptor on the membrane of the T-lymphocytes has a central role in the proliferative T-lymphocyte response that occurred during acute rejection of a transplanted allograft (5). T-lymphocytes constitutively express a receptor with a low affinity for IL-2, consisting of a beta- and a gamma chain. After activation of T-lymphocytes, the alpha chain of the interleukin-2 receptor (IL-2Rα) is co-expressed on their cell membrane. The IL-2Rα associates with the beta- and gamma chain and a receptor with a high affinity for IL-2 is formed (Figure 1). Binding of IL-2 to this receptor results in a (clonal) proliferation of activated T-lymphocytes, leading to an augmentation of the immune response (6). Blockade of the IL-2Rα is known to inhibit the proliferation of activated T-lymphocytes. Monoclonal antibodies against IL-2Rα, derived from mice, increased the survival of transplanted allografts in several animal studies (7). The efficacy of repeated administration of these murine monoclonal antibodies in humans was limited due to the formation of neutralizing antibodies (human anti-mouse antibodies, *HAMA*). This problem was overcome by the development of "chimeric" and "humanized" antibodies; in this procedure the variable IL-2Rα binding region of the murine antibody is combined with the framework of the constant region of a human IgG1 antibody (figure 2). These recombinant IL-2Rα antagonists do not lead to the formation of *HAMA* of clinical importance (8;9). Currently, basiliximab (a chimeric antibody) and daclizumab (a humanized antibody) have been registered in the Netherlands for prevention of acute rejection after organ transplantation.

Pharmacokinetics and –dynamics

The pharmacokinetic characteristics of daclizumab and basiliximab are comparable to human IgG. The plasma half-lives of daclizumab and basiliximab are 11-20 and ±7 days, respectively (human IgG1 ±20 days) (9;10). The concentration of the antibodies needed to achieve complete blockade of IL-2Rα on the lymphocytes is higher for daclizumab than for basiliximab (>0.5-1.0 μg/mL versus >0.2 μg/mL). It is unknown how long blockade of IL-2Rα should be maintained for optimal rejection prophylaxis. Therefore, the optimal dosing regimen is undetermined. Daclizumab has been evaluated in studies using a five-dose regimen (1 mg/kg i.v. every 14 days). With this regimen complete IL-2Rα blockade was achieved for more than 17 weeks (8). Basiliximab was equally effective when used in a two-dose regimen (20 mg i.v. on day 0 and 4), which induced complete IL-2Rα blockade for a shorter period of 4 to 6 weeks (11). If such a shorter duration of IL-2Rα blockade is equally effective in preventing acute rejection episodes, daclizumab might be also effective when administered less frequently. The preliminary results of a study in patients who received a kidney and

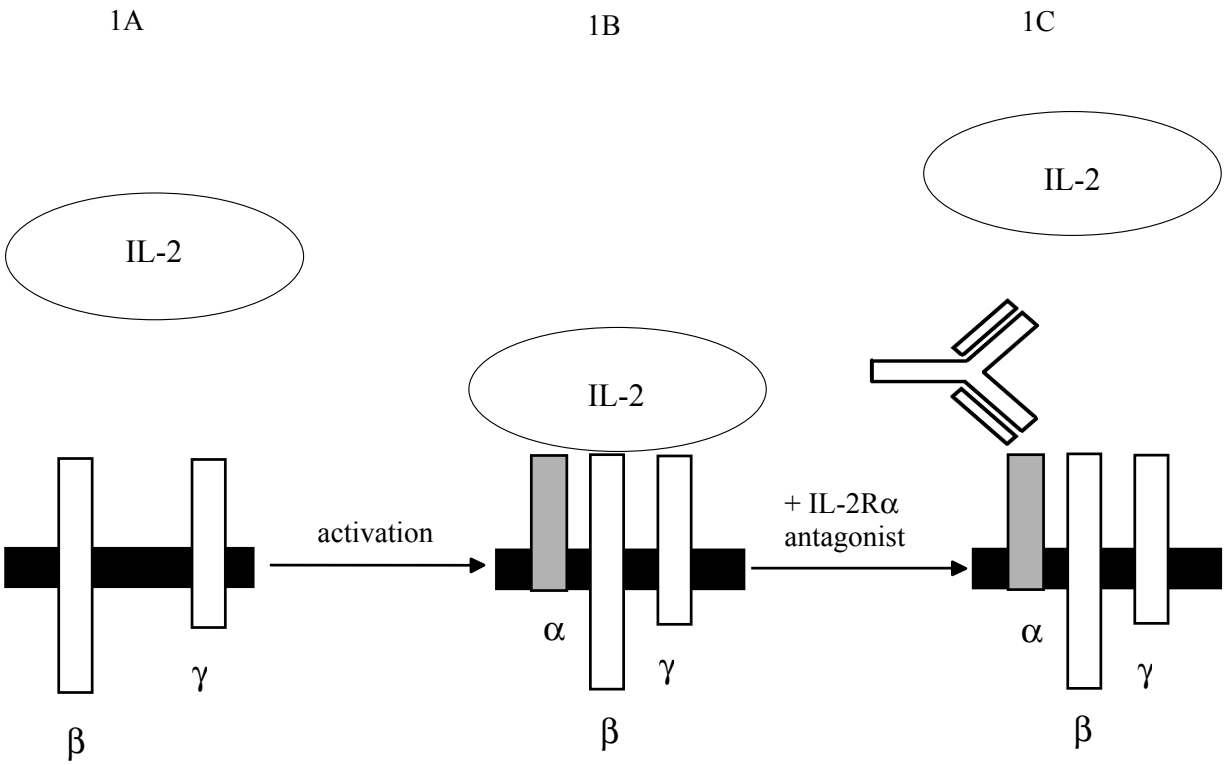

Figure 1. T-lymphocytes constitutively express a receptor with a low affinity for interleukin-2 (IL-2) on their cellular membrane, consisting of a beta and gamma chain (1A). After activation of T-lymphocytes, the alpha chain is co-expressed, and in combination with the beta and gamma chain a receptor with a high affinity for IL-2 is formed (1B). Binding of IL-2 to the IL-2 receptor results in a clonal proliferation of activated T-lymphocytes. Interleukin-2 receptor α antagonists inhibit binding of IL-2 to this high affinity receptor (1C), and the clonal proliferation of activated T-lymphocytes is inhibited.

pancreas allograft simultaneously, suggested comparable efficacy of a two and five-dose regimen of daclizumab (12). Therefore, blockade of IL-2Rα in the first 4-6 weeks after transplantation seems sufficient for adequate rejection prophylaxis. Of note, the number of acute rejection episodes did not increase after resolution of the period of IL-2Rα blockade. In all studies, acute rejection episodes were observed during complete IL-2Rα blockade (11;13), which reflects the potential of cytokines to take over the role of IL-2 as T-cell growth factor during IL-2Rα blockade (14). Since both IL-2Rα antagonists closely resemble human IgG1, no drug interactions are to be expected. Specifically, both agents have no interaction with cyclosporine or mycophenolic acid.

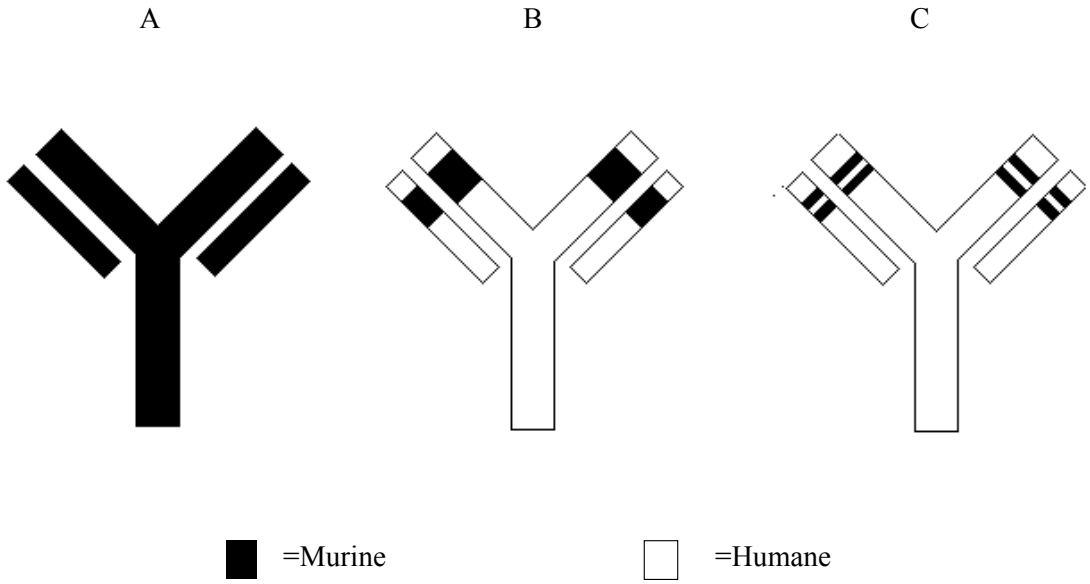

Figure 2. The new generation monoclonal antibodies against the alpha chain of the IL-2 receptor are constructed by combination of the variable, IL-2Rα binding region of the original murine antibody (A), with the framework and constant regions of a human IgG1 antibody. The chimeric antibody basiliximab (B) contains a larger part of the original murine antibody than daclizumab, the humanized antibody (C).

Clinical trials

In four prospective double-blind placebo controlled trials, the effect of addition of daclizumab or basiliximab to a standard immunosuppressive regimen of cyclosporine (CsA) and prednisone (with or without azathioprine) was investigated in more than 1200 recipients of a renal transplant (10;15-17). The design of these four studies was comparable (Table 1). The inclusion to the study was restricted to patients with a low risk for acute rejection (recipients of a first allograft, who had no antibodies against HLA). Induction therapy with ATG was not used. The patients were followed for one year, and analysis was on an intention-to-treat basis.

Acute rejection episodes. In all studies the incidence of biopsy-proven acute rejection was significantly lower in patients treated with the IL-2Rα antibody (Table 1).

In the daclizumab treated patients the incidence of acute rejection decreased by 37-40%, in the basiliximab treated patients by 29-30%. In all studies, the use of IL-2Rα antibody therapy reduced the need for treatment of (steroid-resistant) acute rejection with additional anti-T-

lymphocyte therapy. The cumulative steroid dose in the first year after transplantation was also lower in the IL-2Rα antibody treated group, a logical consequence of the lower rate of acute rejection. In two of the four studies, renal function at six months after transplantation was better in the IL-2Rα antibody treated patients.

Survival. Despite the lower rate of acute rejection in the IL-2Rα antibody treated patients, no differences were found in one-year graft survival. Only one study reported a 5% improve in patient survival (15).

Side effects It is rather remarkable that in the studies presented so far, the use of daclizumab and basiliximab did not lead to an increased number of adverse vents compared with placebo. Especially, the cytokine-release syndrome, a common side effect after treatment with OKT-3, did not occur. Besides, the incidence of (opportunististic) infections or malignancies (e.g. posttransplantation lymphoma) was not increased in any of the four studies. Similar results have been obtained in a study evaluating the use of daclizumab after heart transplantation (18).

Table 1. Results of four clinical trials investigating the effect of addition of daclizumab or basiliximab after renal transplantation

First author(s) (ref)	*Vincenti (10)*	*Nashan, Light (15)*	*Nashan, Moore (16)*	*Kahan (17)*
No of patients	260	275	376	348
No of participating centers	17	19	21	21
Basic immunosuppressive therapy	CsA + AZA + pred	CsA + pred	CsA + pred	CsA + pred
Study medication (versus placebo)	daclizumab	daclizumab	basiliximab	basiliximab
Results versus placebo				
Biopsy-proven acute rejection	22% versus 35%*	28% versus 47%#	30% versus 44%*	35% versus 49%#
Anti T- cell therapy	8% versus 14%	8% versus 16%*	10% versus 23%#	20% versus 30%*
Graft survival (first year)	95% versus 90%	88% versus 83%	88% versus 87%	95% versus 93%
Patient survival (first year)	98% versus 96%	99% versus 94*	95% versus 97%	97% versus 96%
Median GFR at 6 months (ml/min)	55 versus 52	58 versus 51*	52 versus 51	57 versus 52*
No of infections	47% versus 52%	74% versus 72%	85% versus 87%	75% versus 73%
Posttransplantation lymphoma (no of patients)	1 versus 3	0	1 versus 1	0 versus 1

CsA= cyclosporine; AZA= azathioprine; pred= prednisone; GFR= glomerular filtration rate

*$p \leq 0.05$; # $p \leq 0.01$ (IL-2Rα antagonist versus placebo)

Discussion

In conclusion, both daclizumab and basiliximab decrease the incidence of acute rejection after renal transplantation, apparently without causing any side effects. The use of IL-2Rα antibodies did not improve one-year graft survival. Therefore, studies with long-term follow-up have to be awaited to determine if the positive effects on preventing acute rejection episodes will translate in better long-term survival. Studies comparing daclizumab and basiliximab are lacking, and therefore there is no preference for one of the two monoclonal antibodies. Using the advised dosing regimen the costs treating an average person of 70 kg are ± 2.330 Euro's for basiliximab, and ± 5083 Euro's for daclizumab. The lower costs together with the easiness of the dosing regimen of basiliximab are a clear advantage of this drug. However, as stated earlier, it is possible that daclizumab is equally effective in a two-dose regimen. In that case, treatment with daclizumab would be somewhat more cost effective. Currently, the combination of a calcineurine inhibitor (CsA or tacrolimus), mycophenolate mofetil, and prednisone is the standard regimen in most centers in the Netherlands. With this combination, the incidence of acute rejection ranges from 15 to 20% (2), lower than the rejection rate observed in the presented trials with basiliximab and daclizumab. The preliminary results of a phase II double blind randomized trial suggest that the addition of daclizumab to CsA, mycophenolate mofetil (MMF) may further decrease the rejection rate to ±12% (19).

Acute rejection versus side effects. Although a reduction of the rate of acute rejection may seem an important goal of immunosuppressive therapy, the relevance of a low incidence of acute rejection for long-term outcome is unclear. Long-term patient and graft survival are influenced to a major extent by factors not directly related to acute rejection such as cardiovascular disease and malignancies. These factors are dependent on the total immunosuppressive load (in case of malignancies), and specific side effects of the immunosuppressive drugs. Therefore, the important advance of basiliximab and daclizumab is especially the lack of side effects. Current (ongoing) studies address the question whether these monoclonal antibodies can safely replace one of the other, more toxic immunosuppressive drugs. The preliminary results of a phase II open-label study with a calcineurin inhibitor-free immunosuppressive regimen consisting of daclizumab, MMF and prednisone revealed an incidence of acute rejection of 50% (20). Thus the replacement of a calcineurin inhibitor by an IL-2Rα antibody seems to result in a (unacceptable) high risk for acute rejection.

At November 1999, a prospective randomized controlled multi-center study was started to test the hypothesis that the addition of two doses of daclizumab to tacrolimus and mycophenolate mofetil would enable the use of a steroid-free immunosuppressive regimen without affecting the incidence of acute rejection. Four kidney transplant centers in the Netherlands are participating in this study (Nijmegen, Rotterdam, Utrecht, and Maastricht). The results of this study will help to define the position of anti IL-2Rα treatment in transplantation.

Conclusion

Addition of daclizumab or basiliximab, to a regimen with CsA and prednisone results in a decreased incidence of acute rejection in the first year after transplantation, without an increase in adverse events. The impact of this finding on long-term graft and patient survival is unclear. These agents provide a more selective way of immunosuppression, which might allow reducing the dose or avoiding the use of more toxic immunosuppressive drugs.

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Chapter 3

Two doses of daclizumab are sufficient for prolonged IL-2Rα blockade

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Daclizumab, a humanized antibody to the alpha subunit of the IL-2 receptor (IL-2Rα-chain), decreases the incidence of acute rejections after organ transplantation (1;2). After intravenous administration the antibody binds to the IL-2Rα-chain of T lymphocytes, thereby inhibiting the binding of IL-2 and the subsequent transduction of the IL-2 signal. This results in a selective blockade of the activation of antigen-specific T lymphocytes. Blockade of the IL-2Rα-chain, as measured by flow cytometry, is an in vitro parameter of the effectiveness of daclizumab. The current recommendation is to use daclizumab in a regimen consisting of five doses of 1 mg/kg intravenously (on day 0 and week 2, 4, 6 and 8 after transplantation). Using this regimen in combination with cyclosporine, azathioprine, and prednisone, Vincenti et al. (1) observed blockade of the IL-2Rα-chain lasting up to 17 weeks after renal transplantation. In a multicenter study, we aimed to block the IL-2Rα-chain for a period of 10 weeks after renal transplantation by administration of two doses of daclizumab on day 0 and day 10 after transplantation. In case of reappearance of IL-2Rα^{pos} T lymphocytes (as defined by a rise of the CD3^{pos}CD25^{pos} lymphocytes above 4% of the total population) within the first 10 weeks after transplantation, an additional dose of daclizumab was administered. The basic immunosuppressive regimen consisted of a combination of tacrolimus and mycophenolate mofetil. Flow cytometry was performed in whole blood before the infusion of daclizumab and at week 2, 4, 6, 8 and 10 after transplantation in 40 adult recipients of a kidney transplant as described previously (3). Reappearance of IL-2Rα^{pos} T lymphocytes was not found in any of the patients in the first 10 weeks after transplantation, so no additional doses of daclizumab were given. In a subgroup of 24 patients flow cytometry was continued until the IL-2Rα^{pos} T lymphocytes reappeared. In these 24 patients IL-2Rα^{pos} T lymphocytes reappeared at 16 weeks (=median; range 11-30 weeks) following transplantation (figure 1). Five of the 40 (13%) patients experienced a biopsy-proven acute rejection between 4-81 days after transplantation, during a follow-up of 29 weeks (=median; range: 12-48 weeks). Four of these rejections appeared during blockade of the IL-2Rα-chain, which underscores the redundancy of the immune system in activating T lymphocytes (4). Two of these patients were treated with anti-T lymphocyte globulin, because of a steroid resistant acute rejection. There were no immunologically mediated graft losses.

In conclusion, a two-dose regimen of daclizumab in combination with tacrolimus and mycophenolate mofetil results in a blockade of the IL-2Rα-chain during more than 10 weeks after transplantation. The blockade of the IL-2Rα-chain was longer than expected. This might be explained by the co-administration of mycophenolate mofetil, since this drug also

increased the duration of the IL-2Rα-chain blockade when co-administered with basiliximab, another monoclonal antibody to the IL-2Rα-chain (5). The two-dose regimen of daclizumab in combination with tacrolimus and mycophenolate mofetil seems sufficient for adequate immunosuppression as is suggested by the low incidence of acute rejection. The benefits of the two-dose regimen compared with the previously advocated five-dose regimen are a reduction in costs (saving ± 3000 Euro's per patient of 75 kg) and the avoidance of the logistic problems associated with the intravenous administration of daclizumab in the outpatient clinic.

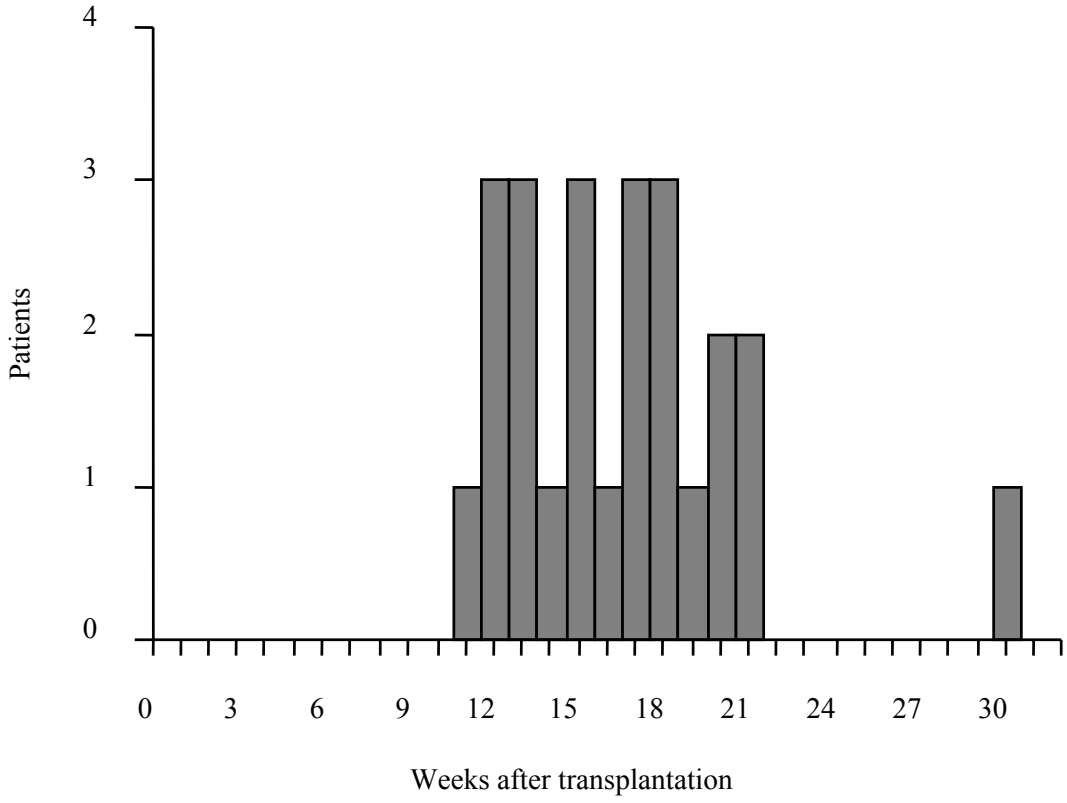

Figure 1. The moment of reappearance of the IL-2Rα^{pos} lymphocytes after transplantation in 24 patients who received two doses of daclizumab (1mg/kg/d i.v.).

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Chapter 4

Decreased renal excretion of soluble interleukin-2 receptor α after treatment with daclizumab

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Abstract

Daclizumab (±150 kD), a humanized mAb against the alpha-chain of the membrane bound IL-2 receptor also binds soluble interleukin-2Rα (sIL-2Rα; ±45 kD), and thus may influence the glomerular filtration of sIL-2Rα. We have studied the influence of daclizumab on the renal excretion of sIL-2Rα in 38 recipients of a renal transplant (32 daclizumab-treated; 6 controls). Soluble IL-2Rα was measured every two weeks after transplantation in serum and urine with Immulite® IL-2R, a solid phase ELISA. In the control population the fractional excretion of sIL-2Rα was relatively constant with a median value of 1.7±0.5%. In daclizumab-treated patients sIL-2Rα was not detectable in the urine immediately after the administration of daclizumab. Soluble IL-2Rα became detectable in the urine at a mean of 8±3 weeks after transplantation. In additional experiments, serum compounds were separated by size-exclusion chromatography and sIL-2Rα was measured in the collected fractions. In the control patients sIL-2Rα was only present in the low-molecular-weight fractions of serum. In contrast, in daclizumab-treated patients evaluated several weeks after transplantation, sIL-2Rα was merely detected in the high-molecular-weight fractions of serum. During follow-up there was a relative shift of sIL-2Rα from the high- to the low-molecular-weight fractions and this coincided with normalization of sIL-2Rα excretion. Daclizumab inhibits the renal excretion of sIL-2Rα by the formation of a complex with sIL-2Rα in serum, which is too large for glomerular filtration. Measurement of urinary sIL-2Rα may provide information on the concentration of anti-IL-2Rα mAb in serum.

Introduction

Interleukin-2 (IL-2) plays a critical role in the proliferative expansion of antigen-stimulated T-cells, and exerts its growth promoting action via specific cell membrane receptors (1). A low affinity receptor for IL-2, consisting of a beta and a gamma chain is constitutively expressed on lymphocytes. Activation of lymphocytes results in co-expression of the alpha chain (IL-2Rα or CD25) leading to the formation of an IL-2 receptor with a high affinity for IL-2 (2). Soluble interleukin-2 receptor alpha (sIL-2Rα, ±45 kD) is the truncated form of the alpha chain of the IL-2 receptor and is derived from proteolytic cleavage of membrane bound IL-2Rα (3;4). Soluble IL-2Rα retains the ability of binding interleukin-2 with a low affinity (5-7). Soluble IL-2Rα is found in the urine and serum of healthy persons, and increased serum concentrations are found in a variety of diseases such as T-cell leukemia, AIDS, autoimmune diseases, and after organ transplantation (3;8). These increased concentrations are the result of an increased production and have been proposed to reflect the tumor burden or the rate of immunologic activation. In recipients of organ transplants, high concentrations were found during acute rejection, CMV infection and after treatment with antithymocyte globulin. Several studies have reported that increased concentrations of sIL-2Rα in serum and urine may be helpful in detecting acute rejection (9-14), although other studies have challenged the usefulness of sIL-2Rα for this purpose (15;16). Normally, 90% of sIL-2Rα is catabolized by the kidneys, a process governed by glomerular filtration and tubular reabsorption (17). A small amount of filtered sIL-2Rα escapes tubular reabsorption and is excreted in the urine. Administration of anti-IL-2Rα antibodies may affect normal renal handling of sIL-2Rα by forming a large complex that cannot be filtered. Indeed, administration of anti-IL-2Rα mAb in mice appeared to inhibit the renal clearance of sIL-2Rα (17). Thus far, the effect of an anti-IL-2Rα mAb on the renal excretion of sIL-2Rα has not been studied in humans. Daclizumab, a humanized monoclonal IgG1 directed against IL-2Rα is a competitive inhibitor of IL-2 (18). Daclizumab has been proven effective for acute rejection prophylaxis after organ transplantation, and preliminary results suggest beneficial effects in the treatment of graft-versus-host disease, uveitis anterior and refractory psoriasis (9;19-22). We investigated the influence of daclizumab on the renal excretion of sIL-2Rα in recipients of a renal transplant. Our hypothesis was that a decline in the excretion of sIL-2Rα reflects the presence of daclizumab in serum. Such a finding might be helpful for therapeutic drug monitoring of daclizumab.

Materials and methods

Study population

The study population consisted of 38 adult patients who received a renal allograft at the University Medical Center Nijmegen, the Netherlands. Patients participated in a prospective randomized controlled trial comparing induction therapy with daclizumab (1 mg/kg i.v.) on day 0 and day 10 after renal transplantation (RTx) with maintenance therapy with prednisone, which was tapered from 0.3 mg/kg/day to zero during the first four months after RTx. Methylprednisolone 100 mg i.v. was given to all patients during the first three days after RTx. The additional standard immunosuppression consisted of tacrolimus and mycophenolate mofetil (750 mg bid). Patients receiving daclizumab ($N = 32$) were compared with prednisone treated controls ($N = 6$).

Analytical methods

In all patients, peripheral blood and urine samples were collected preoperatively and approximately every two weeks during outpatient follow-up visits after RTx. Samples of urine produced at 0, 2, 4, and 6 hours after RTx were collected in six recipients of a renal transplant with immediate function (controls: $N = 3$; daclizumab-treated $N = 3$). Serum was frozen at -20 °C and urine at -70 °C until assays were performed in batches. Two urine samples that contained more than one gram/L protein were excluded from analysis, to preclude that the presence of daclizumab in the urine would interfere with the detection of sIL-2Rα in the urine.

ELISA for measurement of sIL-2Rα

Soluble IL-2Rα was measured with a commercially available automated solid phase, two-site chemiluminescent immunometric assay according to the specifications of the manufacturer (Immulite®; DPC, Los Angeles, CA, USA). This assay employs a murine monoclonal antibody against the IL-2Rα (capture antibody) and a polyclonal anti-IL-2Rα-detecting antibody. The values are expressed in U/mL based on the reference standard supplied by the manufacturer, with limits of detection between 50 and 7200 U/mL.

Separation of low- versus high-molecular-weight serum fraction

Serum compounds were separated by size-exclusion chromatography (23) on a hi-load Superdex 75 column (prep grade 16/60; Amersham Biosciences, Roosendaal, the Netherlands). The Superdex 75 column optimally fractionated serum compounds with a molecular weight of ±3 to 70 kD. Serum compounds with a molecular weight above 70 kD are not optimally fractionated, and are found in the first elution fractions. We have calibrated the column using blue dextran (=V0; ±2000 kD), IgG (±150 kD), albumin (±67 kD), ovalbumin (±43 kD), and chymotrypsinogen (±25 kD), dissolved in buffer. Serum samples of one mL were diluted two times with buffer (0.05 M KH_2PO_4, 0.04 M NaOH, and 0.15 M NaCl; Ph=7.4), applied to the column, and eluted with the earlier mentioned buffer at a flow-rate of one mL/min. Elution fractions of one mL were collected. Soluble IL-2Rα was measured in all separate fractions in one control patient and compared with the elution pattern of a daclizumab-treated patient. In 3 controls and 4 daclizumab-treated patients the respective fractions containing substances with an apparent high molecular weight (fraction 49 to 57) were combined and fractions containing substances with an apparent low molecular weight (fraction 58 to 66) were combined.

Other analysis

Urine and serum specimens were assayed for creatinine concentrations by automated methods. Urinary sIL-2Rα was expressed per millimole of urinary creatinine to correct for urine flow and sampling errors. The fractional excretion (FE) of sIL-2Rα was calculated [sIL-2Rα *urine* (U/mL) x creatinine *serum* (μmol/L)] / [sIL-2Rα *serum* (U/mL*)* x creatinine *urine* (mmol/L) x 1000] and expressed as percentage.

Statistical analysis

Unless otherwise indicated, results are expressed as the mean ± SD. A Student *t* test was performed to compare the maximum serum value of sIL-2Rα between groups. A *P* value less than 0.05 was considered statistically significant. The statistical software SPSS version 10.0 for Windows was used (SPSS, Chicago, IL, USA).

Results

The characteristics of the patients are shown in table 1. In pilot experiments we have measured serum sIL-2Rα levels in the morning and in the afternoon to exclude a daytime rhythm. No differences were found (989 U/mL versus 988 U/mL; $N = 4$). Additionally we have compared the sIL-2Rα/creatinine ratio in morning urine samples with the sIL-2Rα/creatinine ratio in 24-hour urine collected at the same day, and again no major difference was observed (74 U/mmol versus 75 U/mmol; $N = 4$).

Table 1. Patient characteristics and results after transplantation.

	Controls ($N = 6$)	**Daclizumab-treated ($N = 32$)**
Age (years)	47±14	50±16
Male (n)	2	20
LRD/CAD/NHBD (n)	2/3/1	15/12/5
Acute rejection (n)	0	2
CMV infection (n)	1	6
Tacrolimus toxicity (n)	0	3
Acute tubular necrosis (n)	1	6
Serum creatinine[a] (μmol/L)	148±54	152±32

Abbreviations are: LRD, living related donor; CAD, cadaveric donor; NHBD, non-heart beating donor; CMV, cytomegalovirus.

[a] Three months after transplantation

Daclizumab influences the measurement of sIL-2Rα by ELISA

To investigate the effect of daclizumab on the measurement of sIL-2Rα, daclizumab was added in concentrations from 0 to 5.0 μg/mL to a control serum. As shown in Figure 1, addition of increased doses of daclizumab decreased the levels of sIL-2Rα as measured by the Immulite® assay. Thus, the ELISA does not allow quantitative measurement of the total concentration of sIL-2Rα in serum of daclizumab-treated patients. It remained unclear if the ELISA values reflected the concentration of the free sIL-2Rα in serum.

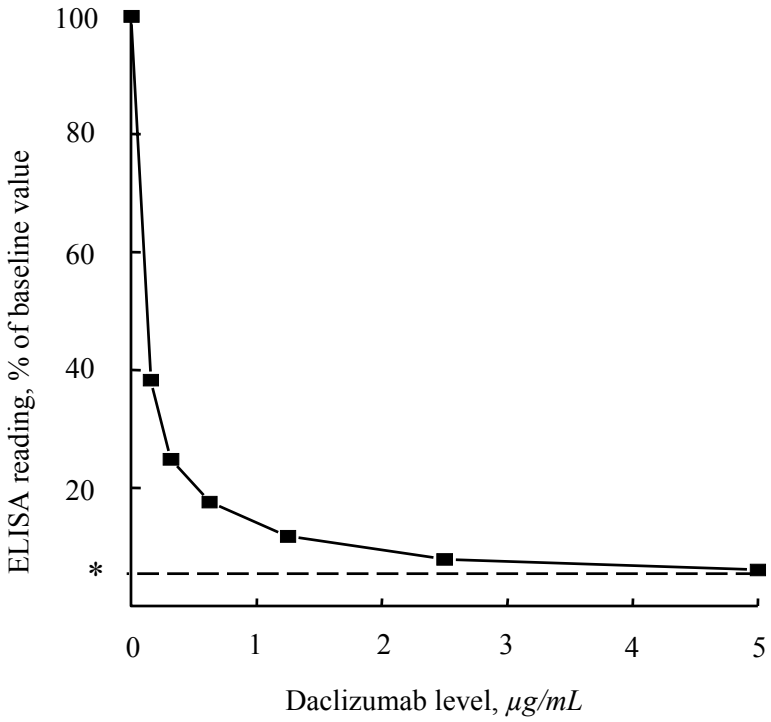

Figure 1. Adding increased doses of daclizumab interferes with the detection of sIL-2Rα by the Immulite® assay, probably by competition with the capture antibody of the ELISA for binding sIL-2Rα. Soluble IL-2Rα expressed as a percentage of the value at baseline (without daclizumab) in serum containing 870 U/mL of sIL-2Rα. *: Limit of detection.

Soluble IL-2Rα values in high- and low-molecular-weight fractions of serum

To determine if the ELISA only detected free sIL-2Rα, additional studies were done using size-exclusion chromatography. As expected, in a control patient sIL-2Rα was detected in the low-molecular-weight serum fractions (Fig. 2). From Figure 2 it appears that the apparent molecular weight of the sIL-2Rα is higher than expected, most likely the result of some changes in physiochemical properties of sIL-2Rα in the presence of serum or formation of dimers. In this control patient, the sum of sIL-2Rα measured in the different fractions was similar to the value measured in unfractionated serum, indicating a good recovery. After addition of daclizumab to a control serum (concentration of sIL-2Rα: 1139 U/mL; final concentration of daclizumab: 0.9 µg/mL), the measured sIL-2Rα value decreased to 160 U/mL. After fractionating the serum, sIL-2Rα was only recovered in the serum fractions with

a high molecular weight (Fig. 2). The sum of sIL-2Rα measured in the fractions was approximately three times higher than the value measured in unfractionated serum, and approached the value measured in the serum before the addition of daclizumab.

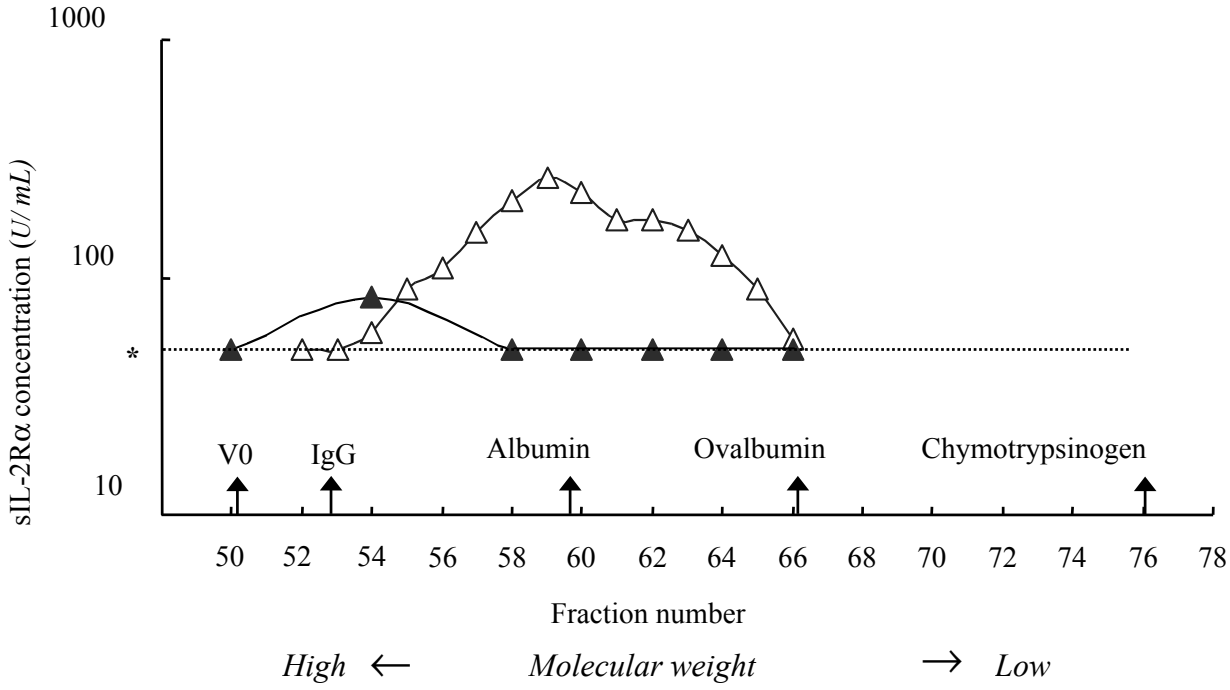

Figure 2. Elution pattern of sIL-2Rα in a control patient (open triangles) showing presence of sIL-2Rα predominantly in the low-molecular-weight serum fractions. After addition of daclizumab (0.9 μg/mL), sIL-2Rα was only detectable in the fractions with a high molecular weight (closed triangles). Soluble IL-2Rα values expressed on log scale; *: Limit of detection = 50 U/mL; Calibration of the column was performed with blue dextran (=V0; ±2000 kD), IgG (±150 kD), albumin (±67 kD), ovalbumin (±43 kD), and chymotrypsinogen (±25 kD).

The effect of dilution on the ELISA measurements

The difference in sIL-2Rα values measured before and after fractionation of daclizumab-containing serum might be related to dilution during the fractionation procedure. Therefore, the effect of dilution of serum on ELISA measurements was studied in vitro. In control patients dilution of the serum did not affect the recovery of sIL-2Rα (i.e. ELISA readings decreased linearly to the dilution step) (Fig. 3). In contrast, in daclizumab-treated patients

ELISA readings of diluted serum samples were higher than expected (Fig. 3). Taken together, it is evident that in the ELISA the capture antibody competes with daclizumab for binding of sIL-2Rα. The amount of sIL-2Rα that is detected by the ELISA therefore depends on the concentration and the affinity for sIL-2Rα of both antibodies. This also explains the increase in recovery of sIL-2Rα upon dilution of daclizumab containing serum. With increased dilution of serum the concentration of daclizumab decreases relative to the (fixed) concentration of the capture antibody of the ELISA, and relatively more sIL-2Rα is measured by the assay due to an increased dissociation of sIL-2Rα in complex with daclizumab.

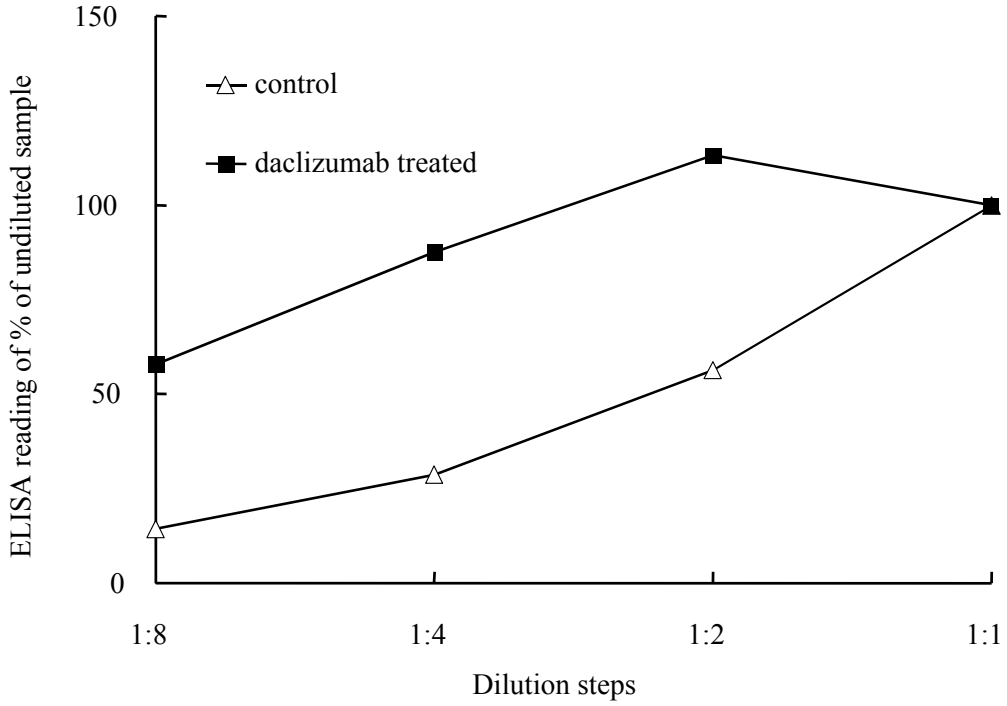

Figure 3. The effect of dilution of serum on the measurement of sIL-2Rα by the ELISA. The values are expressed as a percentage of the value without dilution; (open markers: average of three controls; black markers: average values of serum taken several weeks after transplantation in two daclizumab-treated patients). In control patients, dilution of the serum did not affect the recovery of sIL-2Rα, i.e. ELISA readings decreased linearly to the dilution step. In contrast, in daclizumab-treated patients ELISA readings of diluted serum samples were higher than expected. With increased dilution, the concentration of daclizumab decreased relative to the (fixed) concentration of the capture antibody of the ELISA, and relatively more sIL-2Rα is measured by the assay due to an increased dissociation of the sIL-2Rα-daclizumab complex.

The effect of daclizumab on sIL-2Rα values in the urine and serum

In the control patients the serum concentration of sIL-2Rα decreased in parallel with the recovery of renal function after transplantation and remained stable in the absence of an acute rejection or CMV infection (Fig. 4A). Soluble IL-2Rα was detectable in the urine collected immediately after transplantation (Fig. 5). The fractional excretion of sIL-2Rα was relatively constant at an average of 1.7±0.5%.

There was a characteristic pattern of the measured sIL-2Rα values in the urine and serum in all daclizumab-treated patients (Fig. 4A and B), which was completely different from the control population. Soluble IL-2Rα was undetectable in the first urine collected immediately after the administration of daclizumab (Fig. 5) and urinary sIL-2Rα remained undetectable for an average of 8±3 weeks after transplantation. Thereafter the urinary excretion of sIL-2Rα increased to values observed in control patients. After the administration of daclizumab the serum value of measured sIL-2Rα initially decreased to on average 18±11% of the baseline value. Subsequently the measured sIL-2Rα serum value increased to an average maximum of 130±40% of the baseline value at 15±2 weeks after RTx and subsequently decreased for the second time. The peak serum value of sIL-2Rα was approximately two times higher than the maximum serum values measured in controls after RTx (P <0.01). The pattern of the serum value of sIL-2Rα measured as well as the peak serum value of sIL-2Rα in daclizumab-treated patients was not different between patients experiencing (N = 6) or not experiencing (N = 26) a CMV infection [average peak serum level of sIL-2Rα: 119±44% versus 133±40% of the baseline value (NS)].

Urinary excretion of sIL-2Rα in relation to the presence of sIL-2Rα in different molecular-weight fractions of serum

In three control patients, sIL-2Rα was merely detected in the serum fractions with an apparent low molecular weight (Table 2). In four daclizumab-treated patients analyzed two weeks before the peak serum value of measured sIL-2Rα, sIL-2Rα was predominantly detectable in the high-molecular-weight fractions of serum. The urinary sIL-2Rα concentration was low at that moment. The sum of the values of sIL-2Rα measured in the high and low molecular fractions was up to five times higher than measured in unfractionated serum. This recovery was even higher than described in the in vitro experiment of Figure 2 indicating substantial

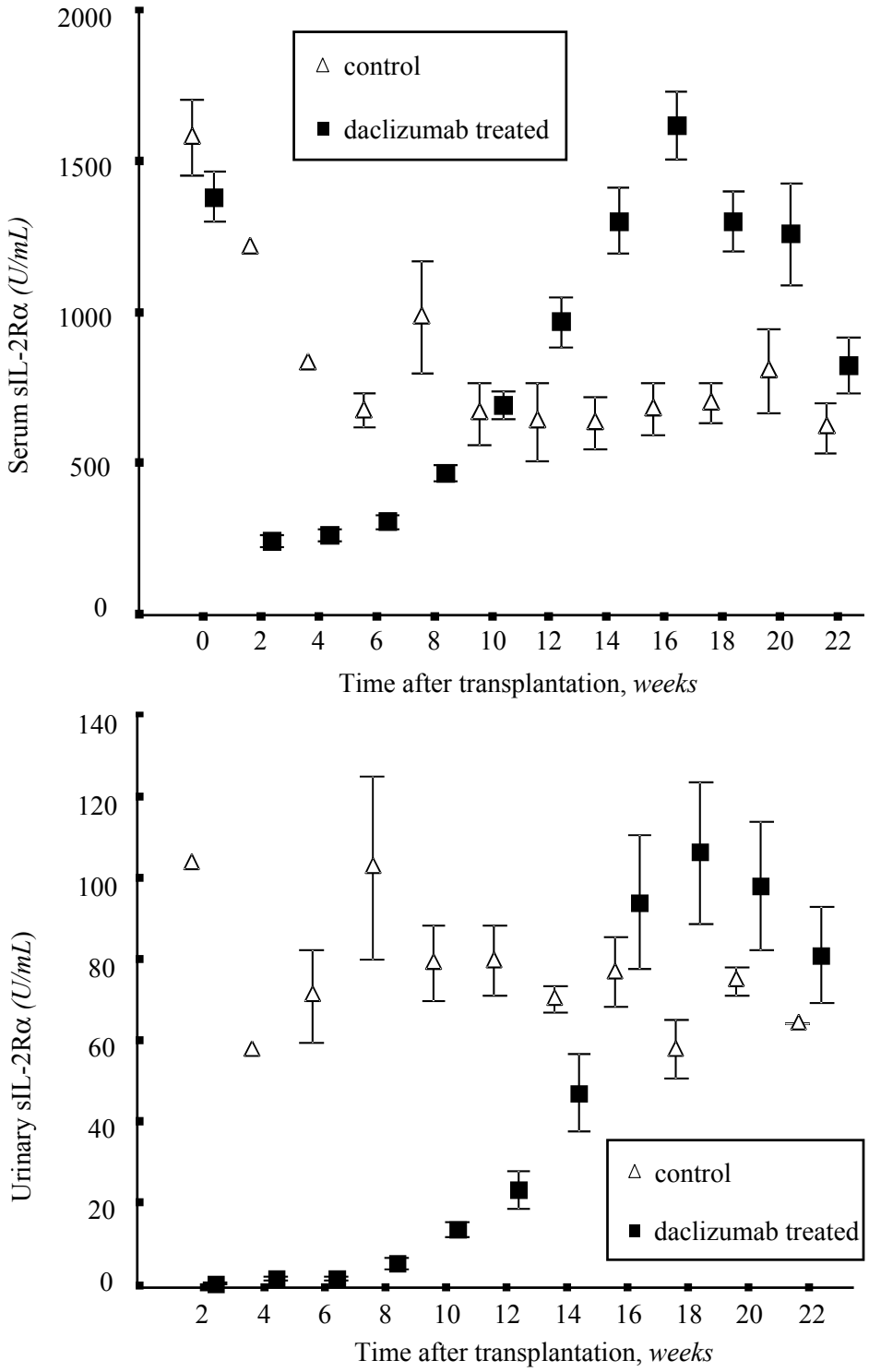

Figure 4A and B. Time course of the mean (±SE) serum (panel A) and urinary (panel B) concentration of sIL-2Rα (adjusted for urinary creatinine) in 6 control patients and 32 daclizumab-treated patients after transplantation. In contrast to the controls, no sIL-2Rα is excreted in daclizumab-treated patients in the first weeks after transplantation.

accumulation of complexes in these daclizumab-treated patients analyzed several weeks after transplantation.

Subsequent analysis in two daclizumab-treated patients with a nearly normalized fractional sIL-2Rα excretion, showed an increase in the proportion of sIL-2Rα measured in the low-molecular-weight fraction of serum (Table 2). As illustrated in Figure 6, a change of the fractional sIL-2Rα excretion towards normal coincides with a relative shift of serum sIL-2Rα from the high- to the low-molecular-weight fractions.

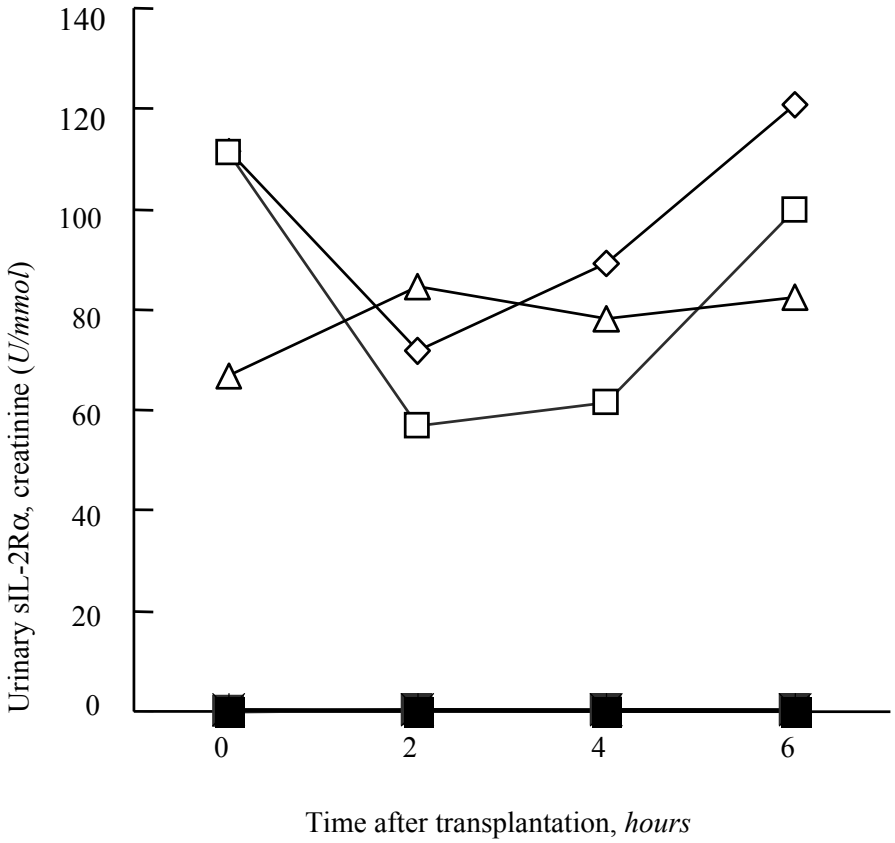

Figure 5. Urinary excretion of sIL-2Rα in three control patients (open markers) and three patients treated with daclizumab (black markers) in the first hours after transplantation. Soluble IL-2Rα was undetectable in all three daclizumab-treated patients.

Table 2. Soluble IL-2Rα measurements in the high- and low-molecular-weight serum fractions and the urinary excretion sIL-2Rα in daclizumab-treated patients and in controls. Values are expressed as ranges.

Measured sIL-2Rα	**Controls (*N* = 3)**	**Daclizumab-treated (*N* = 4)**	
		Before peak (*N* = 4)	**After peak[a] (*N* = 2)**
Low-molecular-weight fraction (U/mL)	606-1272	0-720	612-787
High-molecular-weight fraction (U/mL)	0-420	3612-7056	960-1140
Proportion in low-molecular-weight fraction[b]	75-100%	0-10%	39-40%
Urinary concentration (U/mmol creatinine)	73-168	24-51	88-117
Measured fractional excretion (%)	1.4-2.7	0.3-0.55	1-1.1

[a] In daclizumab-treated patients samples were taken before and after the peak of the measured sIL-2Rα value in serum

[b] Expressed as a percentage of the sum of sIL-2Rα recovered in the low- and high-molecular-weight fractions

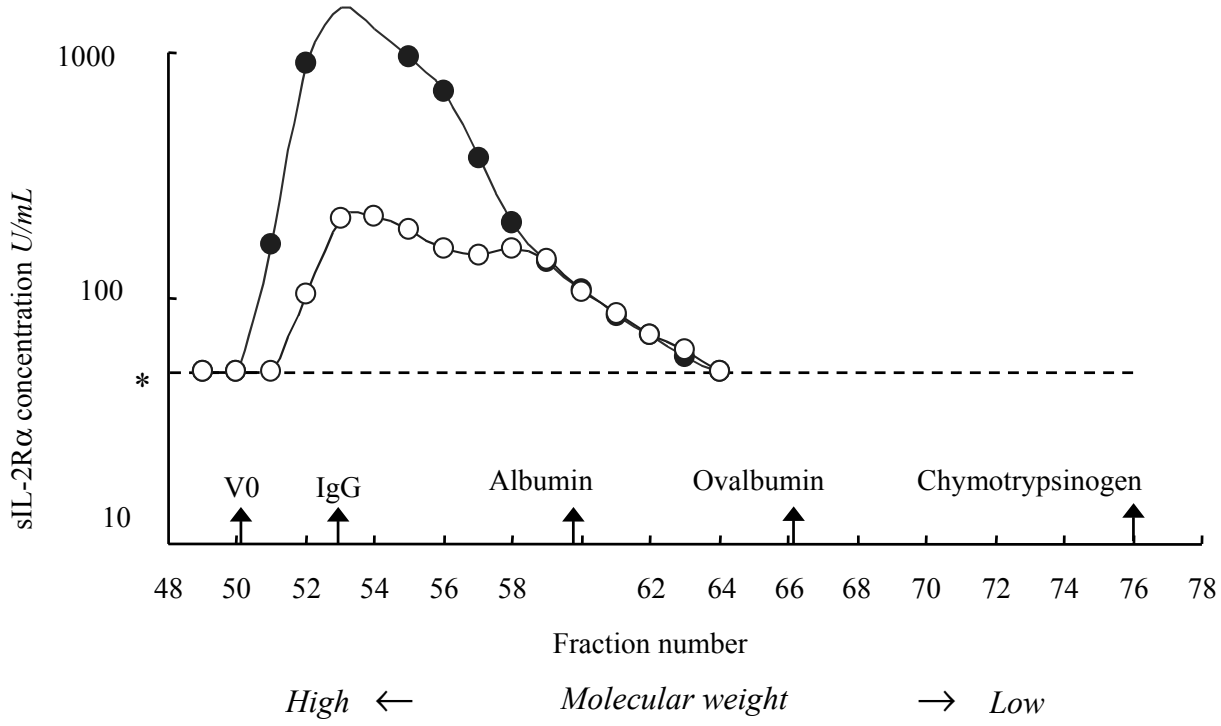

Figure 6. Elution pattern of sIL-2Rα in a daclizumab-treated patient showing presence of sIL-2Rα predominantly in the high-molecular-weight serum fractions (closed circles). The urinary excretion of sIL-2Rα at this stage was 28 U/mmol creatinine. This patient was studied six weeks later (open circles), when the urinary excretion of sIL-2Rα had increased to 88 U/mmol creatinine (fractional excretion 1.0 %). At this time point sIL-2Rα had shifted from the high- to the low-molecular-weight fractions of serum. (Soluble IL-2Rα values expressed on log scale; *: Limit of detection = 50 U/mL); Calibration of the column was performed with blue dextran (=V0; ±2000 kD), IgG (±150 kD), albumin (±67 kD), ovalbumin (±43 kD), and chymotrypsinogen (±25 kD).

Discussion

We have studied the renal handling of sIL-2Rα in renal transplant recipients and specifically evaluated the effect of daclizumab.

Daclizumab is a humanized mAb against the alpha chain of the IL-2 receptor, which is expressed on the membrane of activated peripheral lymphocytes. Our data confirm that in addition to membrane bound IL-2Rα, daclizumab also binds to the circulating sIL-2Rα. Consequently, measurement of circulating sIL-2Rα in daclizumab-treated patients may provide information on serum levels of daclizumab. However, the ELISA used for measuring sIL-2Rα is not able to provide precise information on either free or total sIL-2Rα levels. The detection of sIL-2Rα in the high-molecular-weight fraction and the increased recovery of sIL-2Rα after dilution of serum of daclizumab-treated patients indicate, that the capture antibody of the ELISA and daclizumab compete for binding of sIL-2Rα. Binding of free sIL-2Rα to the capture antibody in a serum sample that contains a small amount of sIL-2Rα in equilibrium with daclizumab-bound sIL-2Rα will result in some additional dissociation of the latter complex. Thus, the measured value of sIL-2Rα will be higher than the original free concentration of sIL-2Rα, but substantially lower than the total amount of sIL-2Rα in serum since the sIL-2Rα-daclizumab complex will not dissociate completely. Unfortunately there is no acceptable alternative for the ELISA to determine the serum concentration of (free) sIL-2Rα during therapy with daclizumab more accurately.

On theoretical grounds, we have made the assumption that the detection of sIL-2Rα in the urine is not influenced by contamination of the urine with daclizumab. Daclizumab is a humanized IgG, and has the same physical properties as IgG (24). The average patient has 50-100 grams IgG. After the infusion of 150 mg of daclizumab (2x 1 mg/kg body weight), ±0.3% of the total IgG content (0.150/ (50 +0.15) x 100%) consists of daclizumab. The average concentration of IgG in the urine is 8 mg/L (25). Assuming an intact glomerular barrier, the peak concentration of daclizumab in the urine is ±0.3 % x 8 mg/L = 0.02 µg/mL. Considering the dose-interference curve of Figure 1, it is unlikely that such a low concentration of daclizumab interferes in an important way with the detection of sIL-2Rα in the urine. So spilling of daclizumab in the urine with subsequent inhibition of the ELISA is unlikely in the absence of a (large) glomerular leak.

In the control population, the serum concentration of sIL-2Rα decreased after transplantation compared with the value before transplantation, which is probably explained by the recovery of renal function leading to the elimination of sIL-2Rα (Fig. 4A) (9;13;15). In accordance to others, we found a relatively low fractional excretion of 1.7±0.5%, which suggests tubular reabsorption and subsequent catabolism of sIL-2Rα (14).

In daclizumab-treated patients however, the renal excretion of sIL-2Rα was temporarily completely abolished. This effect was noted immediately after the administration of daclizumab and lasted up to an average of 8 weeks after RTx. By separating the serum in a low- and a high-molecular-weight fraction with size exclusion chromatography, we could relate the urinary excretion of sIL-2Rα to the ratio of bound and free sIL-2Rα in the serum at different time points. Our results showed that the urinary excretion of sIL-2Rα was low when most of sIL-2Rα was bound to daclizumab (= present in the high-molecular-weight fractions), whereas the urinary and fractional excretion of sIL-2Rα restored to a normal value in parallel with a relative increase of free sIL-2Rα. In other words, formation of a complex of daclizumab and sIL-2Rα (sIL-2Rα /anti-IL-2Rα mAb complex: ±200 to 240 kD) that is too large for glomerular filtration abrogates the metabolism and urinary excretion of sIL-2Rα during therapy of daclizumab. Although the ELISA underestimates total sIL-2Rα levels in serum of daclizumab-treated patients, higher values were reached than in controls. This increase in the sIL-2Rα values after treatment with daclizumab indicates accumulation of complexes of sIL-2Rα and daclizumab. These results confirm the findings of Junghans et al who also found a decreased glomerular filtration and subsequently decreased renal catabolism in combination with increased serum levels of total sIL-2Rα during anti-IL-2Rα mAb-directed treatment of mice (17).

Monitoring of sIL-2Rα in the serum or urine seems useless for predicting immunologic activation during anti-IL-2Rα-directed mAb therapy. Several patients in our study experienced a CMV infection. We could not detect an influence of this stimulus for shedding of sIL-2Rα on the pattern of sIL-2Rα values measured in the urine or serum. Apparently, the influence of daclizumab on the renal excretion of sIL-2Rα is a more important determinant of the sIL-2Rα concentration than the increased shedding of sIL-2Rα associated with a CMV

infection. The same might be true for acute rejection, but the proportion of patients experiencing an acute rejection was too small for firm conclusions.
On the other hand, the urinary sIL-2Rα concentration reflects the amount of free sIL-2Rα in the serum, which probably is inversely correlated to the concentration of the anti-IL-2Rα mAb. Therefore, measuring the urinary excretion of sIL-2Rα may be useful to make sure that the concentration of daclizumab is high enough as not to allow detection of sIL-2Rα in the urine. The clinical relevance of this finding, however, is unclear and remains to be determined. Measurement of sIL-2Rα by an automated ELISA has several advantages above the alternative ways of therapeutic drug monitoring of daclizumab like measurement of the concentration of daclizumab (26) and flow cytometric analysis of IL-2Rα^{pos} lymphocytes. It is easier, can be postponed to another more convenient day, and requires less qualified personnel. Prospective studies are needed to compare the methods for the assessment of circulating anti-IL-2Rα mAbs.

We expect that these results can be extrapolated to therapy with other mAb directed against the IL-2Rα chain. The structure of sIL-2Rα is very similar to that of membrane-bound IL-2Rα, with similar epitopes (6). The key factor of the change in catabolism of sIL-2Rα is the ability of a mAb to bind sIL-2Rα with subsequent formation of a complex of the mAb and sIL-2Rα, which is too large to be filtered by the glomerulus. Preliminary results have shown a comparable pattern of the serum concentration of sIL-2Rα after treatment with another IL-2Rα-blocking mAb, basiliximab (27).

In conclusion, daclizumab inhibited the renal excretion of sIL-2Rα. Measurement of the urinary concentration of sIL-2Rα may be useful to make sure that enough anti-IL-2Rα-directed mAb is present in the circulation to inhibit filtration of sIL-2Rα. The clinical relevance of this finding is unclear. Currently, we are comparing the value of monitoring of sIl-2Rα in the urine with flow cytometric analysis of IL-2Rα^{pos} lymphocytes after treatment with daclizumab in a large cohort of patients.

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Chapter 5

The fractional excretion of soluble IL-2 receptor α is an excellent predictor of the IL-2 receptor α status after treatment with daclizumab

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Abstract

Daclizumab is a humanized monoclonal antibody against the alpha chain of the IL-2 receptor (IL-2Rα). We previously have shown, that the urinary excretion of soluble IL-2Rα (sIL-2Rα) is dependent on the presence of daclizumab in serum. We investigated whether the IL-2Rα status, as assessed by flow cytometric analysis is reflected by the concentration of sIL-2Rα in the urine and serum. 272 measurements were performed in 46 recipients of a renal transplant, who were treated with daclizumab in combination with tacrolimus and mycophenolate mofetil. Soluble IL-2Rα was measured in urine and serum with Immulite® IL-2R, a solid phase ELISA. Complete blockade of the IL-2Rα was defined as the presence of less than 5% IL-2Rα^{pos} lymphocytes in the CD3^{pos} population. Receiver-Operating Characteristic curve (ROC) analysis was done to evaluate the performance of serum and/or urine sIL-2Rα in predicting IL-2Rα blockade. The calculated fractional excretion of sIL-2Rα proved to be an excellent predictor of the blockade of the IL-2Rα (ROC analysis area under the curve: 0.95±0.01). A calculated fractional excretion of sIL-2Rα lower than 0.5 percent had a specificity of 100 percent and a sensitivity of 75 percent for the assessment of blockade of the IL-2Rα. In conclusion, blockade of the IL-2Rα after treatment with daclizumab can reliably be assessed by calculation of the fractional excretion of sIL-2Rα. This method is easier to use compared with the flow cytometric analysis of IL-2Rα^{pos} lymphocytes.

Introduction

Daclizumab, a humanized monoclonal IgG1 directed against the alpha chain of the interleukin-2 receptor (IL-2Rα or CD25) is a competitive inhibitor of interleukin-2 (IL-2) (1). Daclizumab has been proven effective for acute rejection prophylaxis after organ transplantation and preliminary results suggest beneficial effects in the treatment of graft-versus-host disease, uveitis anterior and refractory psoriasis (2-5). The optimal duration of IL-2Rα blockade with daclizumab has not been determined yet. Currently, the most widely applied method to measure blockade of the IL-2Rα receptor is flow cytometry (2,5-7). Measurement of the serum concentration of daclizumab (8) is also informative, but has only been performed in highly specialized centers. A serum concentration of daclizumab above 1 μg/ml was associated with blockade of the IL-2Rα (9).

Soluble IL-2Rα (sIL-2Rα) is the truncated form of membrane bound IL-2Rα and is released by the proteolytic cleavage after activation of lymphocytes (10). Normally 90% of sIL-2Rα is catabolized by the kidneys, a process governed by glomerular filtration and tubular reabsorption (11). A small amount of filtered sIL-2Rα escapes tubular reabsorption and is excreted in the urine. Administration of anti-IL-2Rα antibodies in mice appeared to inhibit the renal clearance of sIL-2Rα by forming a large complex that cannot be filtered (11). We have confirmed this finding in renal transplant recipients treated with daclizumab (12). After treatment with daclizumab the urinary excretion of sIL-2Rα was temporarily completely abolished (12). Measurement of the urinary concentration of sIL-2Rα may therefore be useful to make sure that enough daclizumab is present in the circulation. A commercial automated ELISA is available which allows easy and reliable measurement of sIL-2Rα. We investigated whether the level of IL-2Rα blockade as reflected by flow cytometric analysis after treatment with daclizumab can be assessed by measuring sIL-2Rα in serum and urine.

Materials and methods

Study population

The study population consisted of 46 adult patients who received a renal allograft at the University Medical Center Nijmegen, the Netherlands and were treated with daclizumab [1 mg/kg intravenously on day 0 and day 10 after renal transplantation (RTx)]. Patients participated in a prospective randomized controlled trial comparing induction therapy with daclizumab with maintenance therapy with prednisone during the first four months after transplantation (13). Thirteen recipients of a renal transplant who were not treated with daclizumab were used as controls. All patients received prednisolone 100 mg intravenously during the first three days after RTx. The standard maintenance immunosuppression consisted of tacrolimus and mycophenolate mofetil (750 mg bid).

Sample collection

In 30 daclizumab-treated patients and 5 controls peripheral blood and urine samples were collected every two weeks during outpatient follow-up visits after RTx. In the other 16 patients samples were collected at random in the first year after transplantation. Serum was frozen at -20 °C, and because of degradation of proteins in urine stored at -20 °C (14), urine was frozen at -70 °C.

sIL-2Rα measurement

Soluble IL-2Rα was measured with a commercially available, automated solid phase, two-site chemiluminescent immunometric assay according to the specifications of the manufacturer (Immulite®; DPC, Los Angeles, CA, USA). Assays were performed in batches within 4 weeks after sample collection. The average (±SD) recovery of sIL-2Rα in 5 serum samples stored at -20 °C for two months, and in 8 urine samples stored for 4 weeks at -70 °C was 97±7% and 99±13% respectively, so there was no evidence of degradation of sIL-2Rα during storage. The assay employs a murine monoclonal antibody against the sIL-2Rα (capture antibody) and a second polyclonal anti-IL-2Rα antibody. The values are expressed in U/mL based on the reference standard supplied by the manufacturer, with limits of detection between 50 and 7200 U/ml. In the presence of daclizumab in the serum, the assay measures both free sIL-2Rα, and in part sIL-2Rα in complex with daclizumab (12).

Flow cytometric analysis

The presence and expression level of IL-2Rα on lymphocytes was measured in venous blood samples, collected in EDTA tubes at the same times as samples for ELISA measurements. All procedures were performed at room temperature within 24 hours after collection of blood. Whole blood (100 µL) was incubated for 30 minutes in the dark with 5 µL of the mAb anti-CD45 (clone J.33, Beckman Coulter) conjugated to phycoerythrin-cyanin 5.1 (PC5), 10 µL of the mAb anti-CD3 (clone UCHT1, Beckman Coulter) conjugated to fluorescein isothiocyanate (FITC), and 20 µL of the mAb anti-CD25 (clone 2A3, Beckton & Dickinson) conjugated to phycoerythrin (PE) or as a "negative" control 20 µL of the mAb anti-CD4 (clone 13B8.2, Beckman Coulter) conjugated to PE. Subsequently, Facs Lysing Solution (2 ml; Beckton & Dickinson) was added to each sample, mixed well and incubated for 10 minutes in the dark. Samples were centrifuged for 5 minutes at 1400 RPM in a Sorval RC 3B. Cells were washed two times in a phosphate-buffered saline solution (pH 7.4) containing 1% BSA and 0.1% sodium azide. Finally the pellet was resuspended in 500 µL phosphate-buffered saline solution (pH 7.4) containing 1% BSA and 0.1% sodium azide and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA).
The lymphocyte population was selected by gating on the side and forward scatter and for $CD45^{pos}$ cells (loss of debris). There is no clear separation between the $CD3^{pos}CD25^{pos}$ and the $CD3^{pos}CD25^{neg}$ cells (Figure 1). Therefore, we used the mAb anti-CD4-PE as an isotype control to define the cut-off value for CD25-PE^{pos} and CD25-PE^{neg} cells (Figure 1). This cut-off value was subsequently used to determine the presence and expression level of IL-2Rα on lymphocytes. Since the anti-CD25 mAb binds to the same epitope as daclizumab, cells that are saturated by daclizumab are not recognized. Complete blockade of the IL-2Rα was defined as the presence of less than 5% $CD25^{pos}$ lymphocytes in the $CD3^{pos}$ population.

Other analysis

Urine and serum specimens were assayed for creatinine concentrations by automated methods. Urinary sIL-2Rα was expressed per mmol of urinary creatinine to correct for urine flow and sampling errors. The fractional excretion (FE) of sIL-2Rα was calculated by the formula: [sIL-2Rα *urine* (U/mL) x creatinine *serum* (µmol/L)] / [sIL-2Rα *serum* (U/mL*)* x creatinine *urine* (mmol/L) x 1000] and expressed as percentage.

Statistical analyses

Values are expressed as means ± SD, unless indicated otherwise. Non-parametric tests were used to compare values during IL-2Rα blockade with values after disappearance of IL-2Rα

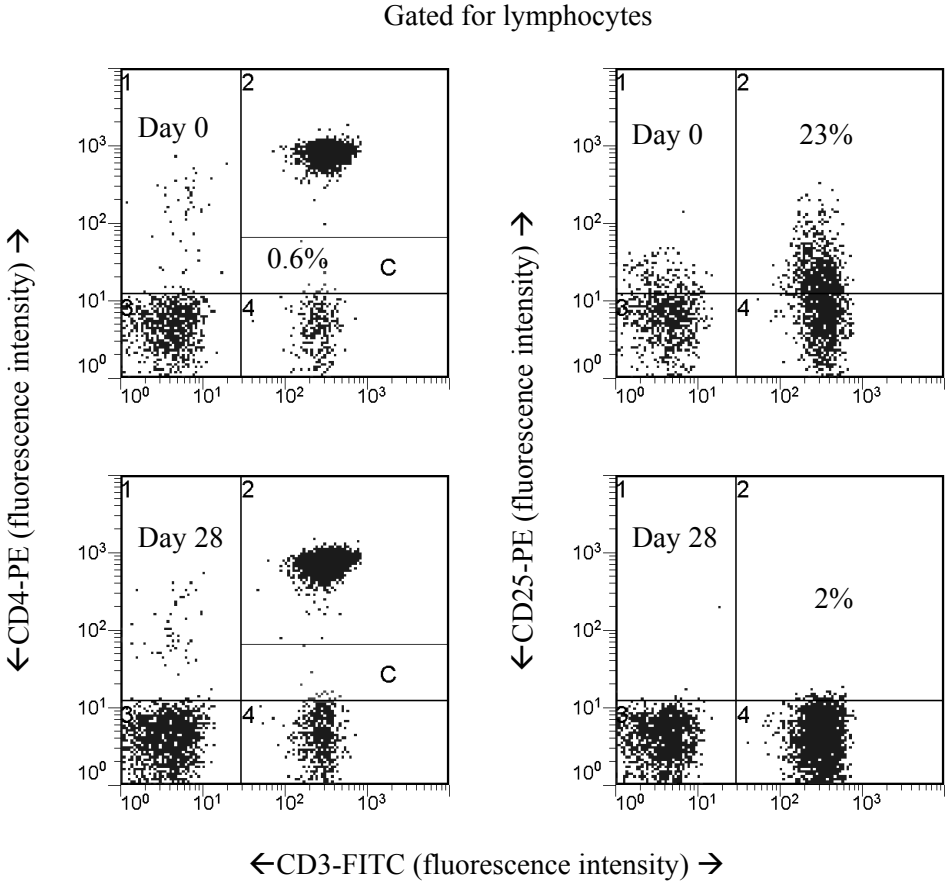


Figure 1. Flow cytometric analysis of peripheral blood lymphocytes of a patient before and 28 days after treatment with daclizumab. The dot plots are gated on lymphocytes. The mAb anti-CD4-PE was used as an isotype control to define the cut-off value for CD25-PE ^{pos} and CD25-PE ^{neg} cells. Quadrant 4 defining the negative population was set in such a way that gate C contained less than 1% of $CD4^{pos}$ cells. Following treatment with daclizumab, the percentage of $CD25^{pos}$ cells decreased from 23% to 2% of the $CD3^{pos}$ population in this patient.

blockade in daclizumab-treated patients or with values in controls not treated with daclizumab. Receiver-Operating Characteristic curve (ROC) analysis (15) was performed with different parameters (urinary sIL-2Rα concentration, and the calculated fractional excretion of sIL-2Rα) for the prediction of the IL-2Rα status as assessed with flow cytometric analysis in daclizumab-treated patients only. With ROC analysis the sensitivity and 1-specificity of the complete spectrum of values of these parameters to predict the IL-2Rα status are displayed, and an area under the curve was calculated. A test with a perfect discriminative power has a sensitivity and specificity of 100% with an area under the curve of 1.

Results

Characteristics of the patients are given in Table 1. Two hundred seventy-two measurements (190 during and 82 after disappearance of IL-2Rα blockade) were performed in 46 daclizumab-treated patients (median 7 measurements per patient; range 2-13) and 61 measurements in 13 controls (median 5 measurements per patient; range 2-12). Serum levels of sIL-2Rα showed a typical pattern with a rapid decrease immediately after the first dose of daclizumab, followed by a gradual increase (12). Treatment with daclizumab profoundly affected the urinary excretion of sIL-2Rα, reflected in a decrease of the urinary excretion of sIL-2Rα during IL-2Rα blockade (Figure 2). The urinary excretion of sIL-2Rα normalized as soon as plasma daclizumab concentrations had decreased to ineffective levels as indicated by the reappearance of $CD25^{pos}$ lymphocytes (Figure 2). ROC analysis based on the complete set of measurements in daclizumab-treated patients (Figure 3), indicated that both the urinary concentration of sIL-2Rα and the calculated fractional excretion of sIL-2Rα were accurate for the assessment of IL-2Rα blockade, with the calculated fractional excretion being somewhat better. Limiting the ROC analysis to two measurements per daclizumab-treated patient (one measurement during IL-2Rα blockade and one after disappearance of IL2Rα blockade) revealed similar but slightly weaker results (AUC for urinary concentration: 0.74±0.06; AUC for calculated fractional excretion: 0.93±0.03). Table 2 lists the sensitivities, specificities, and positive and negative predictive values for various cut-off values of the two parameters using flow cytometric analysis as the gold standard to assess IL-2Rα blockade. Using a value of 0.5% for the calculated fractional excretion as cut-off for predicting IL-2Rα blockade, the specificity was 100% and the sensitivity 75%. Thus, IL-2Rα blockade as assessed by flow cytometric analysis was present at all times when the calculated fractional excretion was below 0.5% (Table 3). A similar specificity was observed when using 14 U/mmol creatinine as cut-off value for the urinary concentration of sIL-2Rα, however the sensitivity was less. In a subgroup of 30 daclizumab-treated patients urine and serum samples were collected every two weeks after RTx. The duration of IL-2Rα blockade as assessed by flow cytometric analysis was 15 weeks (median; range: 10-22 weeks). Six of the subgroup of 30 patients (20%) experienced an acute rejection and were treated with methylprednisolone intravenously. These rejection episodes were diagnosed at a median of 10 days (range 6-24 days) after RTx. So all rejection episodes occurred during IL-2Rα blockade. One patient

however had a transient increase in the calculated fractional excretion of sIL-2Rα up to 1.8 percent 11 days after treatment for an acute rejection. No transient increase in the (calculated fractional) excretion of sIL-2Rα above the threshold values mentioned was found in patients experiencing a CMV infection, tacrolimus toxicity or acute tubular necrosis.

We estimated the duration of IL-2Rα blockade for two defined cut-off values with exclusion of the measurements with a transient increased excretion of sIL-2Rα during the episode of acute rejection. Using a cut-off value of 14 U/mmol creatinine for the urinary concentration of sIL-2Rα, the blockade of the IL-2Rα was estimated to persist for a median of 9 weeks (range 3-15 weeks). Using a cut-off value of 0.5 percent for the calculated fractional excretion of sIL-2Rα, the duration of the blockade of the IL-2Rα was estimated to persist for a median of 12 weeks (range: 4-20 weeks).

Table 1. Characteristics of the 46 recipients of a renal transplant treated with daclizumab and 13 controls, who were not treated with daclizumab.

	Daclizumab (*N* = 46)	**Control (*N* = 13)**
Age (years)	48±14	48±14
Male (n; %)	36 (78)	5 (39)
LRD/CAD/NHBD (n)[a]	20/20/6	6/5/2
Acute rejection (n; %)	10 (22)	2 (15)
Steroid resistant acute rejection (n; %)	2 (4)	0
CMV infection (n; %)	10 (22)	1 (8)
Tacrolimus toxicity (n; %)	9 (20)	1 (8)
Acute tubular necrosis (n; %)	5 (11)	1 (8)
Serum creatinine [a] (μmol/L)	147±32	147±49

Abbreviations are: LRD, living related donor; CAD, cadaveric donor; NHBD, non-heartbeating donor; CMV, cytomegalovirus.

[a] Three months after transplantation

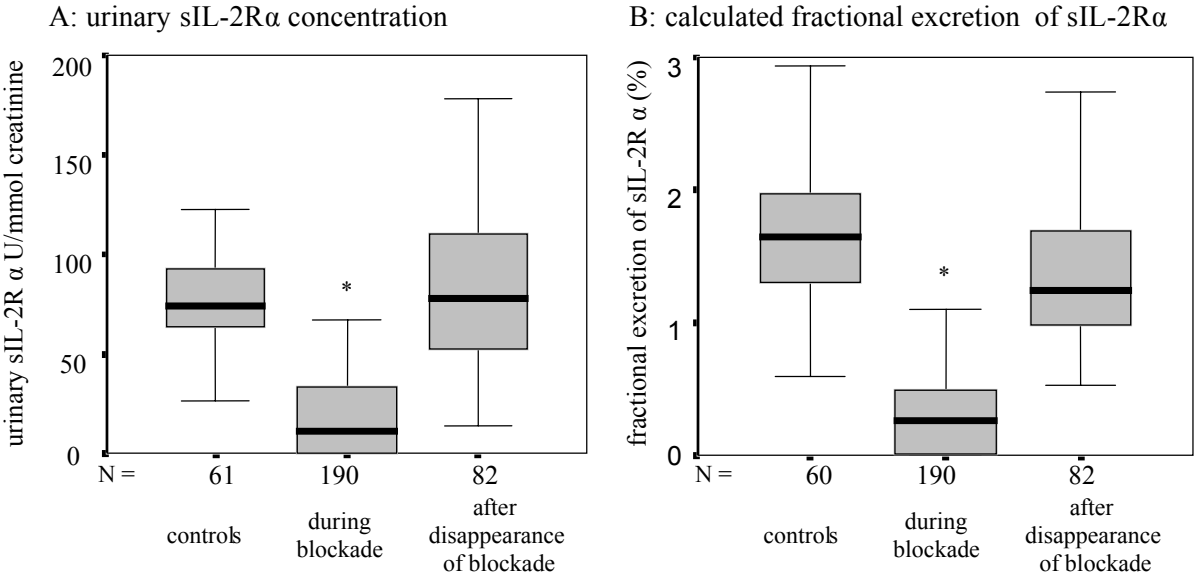
Table 2. Sensitivities, specificities, positive and negative predictive values for various cut-off values of the urinary concentration of sIL-2Rα and the calculated fractional excretion of sIL-2Rα for predicting IL-2Rα blockade after treatment with daclizumab. Flow cytometric analysis was used as the gold standard.

Soluble IL-2Rα	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Urinary concentration (U/mmol creatinine)	Percentage (95 percent confidence interval)			
< 14	58 (50-65)	100 (96-100)	100 (97-100)	51 (43-59)
< 35	75 (68-81)	98 (91-100)	99 (95-100)	65 (56-73)
< 50	81 (74-86)	80 (70-88)	91 (85-95)	64 (54-73)
Calculated fractional excretion				
< 0.5%	75 (68-81)	100 (96-100)	100 (97-100)	64 (55-72)
< 0.6%	85 (79-90)	96 (90-99)	98 (97-100)	73 (64-81)
< 0.7%	88 (83-93)	95 (88-99)	98 (94-99)	78 (69-86)
< 0.8%	90 (85-94)	91 (83-96)	96 (92-98)	80 (70-87)

Table 3. Actual number of measurements with a calculated fractional excretion of sIL-2Rα lower or greater than 0.5 percent in relation to the IL-2Rα status in 46 daclizumab-treated recipients of a renal transplant. IL-2Rα blockade as assessed by flow cytometic analysis was present at all times when the calculated fractional excretion was below 0.5%.

	IL-2Rα blockade[a] ?	
calculated fractional excretion of sIl-2Rα <0.5%?	**present**	**absent**
present	143	0
absent	47	82

[a] As assessed by flow cytometric analysis



*P < 0.001 versus after disappearance of blockade and controls (Mann-Whitney test)

Figure 2. Box plots showing median levels of the urinary sIL-2Rα concentration (Figure 2A) and the calculated fractional excretion of sIL-2Rα (Figure 2B) in 46 daclizumab-treated recipients of a renal transplant in relation to the IL-2Rα status and in13 controls. Boxes show interquartile (25-75%) ranges and error bars represent highest and lowest values. *N* = number of measurements.

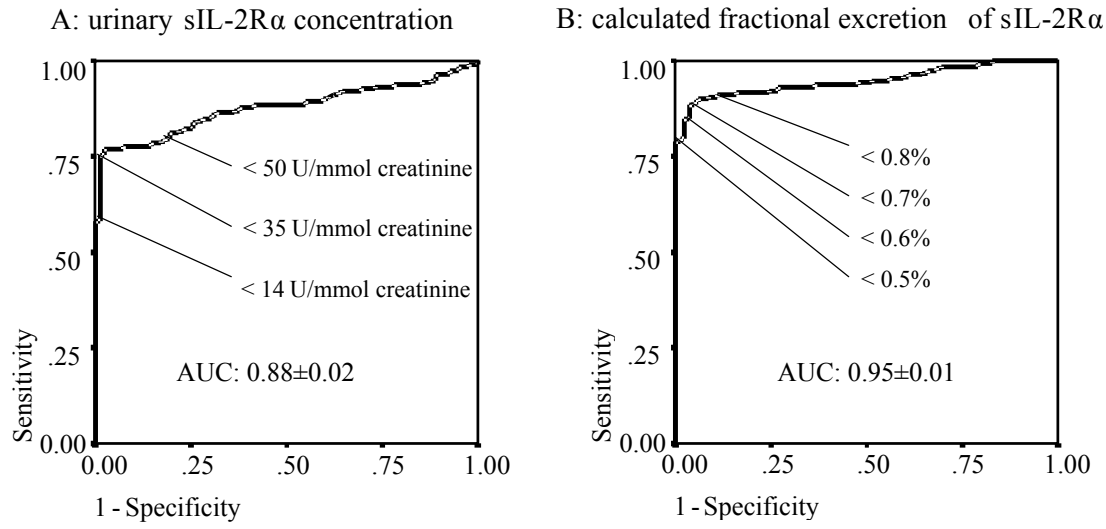


Figure 3. Receiver-Operating Characteristic Curves for the urinary concentration (Figure 3A) and the calculated fractional excretion of sIL-2Rα (Figure 3B) for predicting IL-2Rα blockade after treatment with daclizumab using flow cytometric analysis as the gold standard.

Discussion

We have assessed the value of serum and urine sIL-2Rα in predicting complete IL-2Rα blockade after treatment with daclizumab. We have prospectively studied 46 recipients of a renal transplant who received treatment with daclizumab. The main finding is that both the urinary concentration and the calculated fractional excretion of sIL-2Rα are excellent predictors of the IL-2Rα status after treatment with daclizumab.

The excellent performance of these parameters for the prediction of the IL-2Rα status, is explained by the fact that both the renal filtration of sIL-2Rα and the blockade of membrane bound IL-2Rα depend on the concentration of daclizumab (11,12). Daclizumab temporarily inhibits the renal excretion of sIL-2Rα by binding sIL-2Rα, leading to the formation of a complex that is too large to be filtered and excreted by the kidney. If the concentration of daclizumab decreases, free sIL-2Rα will become present again. Free sIL-2Rα is filtered by the glomerulus and subsequently excreted in the urine. These physiological processes explain that normalization of the calculated fractional excretion of sIL-2Rα coincides with the reappearance of IL-2Rα^{pos} lymphocytes.

Since the above-mentioned mechanism also applies for other IL-2Rα binding mAbs, it seems reasonable to expect that similar results will be found with other IL-2Rα binding mAbs like basiliximab. Measurement of sIL-2Rα by an automated ELISA has several advantages compared to the alternative ways of therapeutic drug monitoring of daclizumab like measurement of the concentration of daclizumab (8) and flow cytometric analysis of IL-2Rα^{pos} lymphocytes. It is relatively easy, and does not require highly qualified personnel.

It has to be noticed that the ELISA used for measuring sIL-2Rα is not able to provide precise information on either free or total sIL-2Rα levels in serum in the presence of daclizumab (12). Both the capture antibody of the ELISA and daclizumab compete for binding to sIL-2Rα. Binding of free sIL-2Rα to the capture antibody in a serum sample that contains a small amount of sIL-2Rα in equilibrium with daclizumab-bound sIL-2Rα will result in some additional dissociation of the latter complex. Thus the measured value of sIL-2Rα will be higher than the original free concentration of sIL-2Rα but lower than the total amount of sIL-2Rα in serum since the daclizumab-sIL-2Rα complex does not dissociate completely (12). Since the daclizumab-sIL-2Rα complex is too large for glomerular filtration, the ELISA measurement in urine samples regards only free sIL-2Rα.

The better performance of the calculated fractional excretion of sIL-2Rα compared with the urinary concentration for the prediction of the IL-2Rα status is a lucky consequence of the overestimation of the serum concentration of free sIL-2Rα by ELISA during the presence of daclizumab in serum. This overestimation of the serum levels of free sIL-2Rα, only occurring during the presence of daclizumab in the serum, results in a lower value for the calculated fractional excretion during IL-2Rα blockade, which enables more accurate classification of the IL-2Rα status. In fact, the increase in the measured serum concentration of free sIL-2Rα is used as a surrogate marker for the presence of daclizumab. In the calculation of the fractional excretion this information is combined with the diminished urinary excretion of sIL-2Rα.
In one of six patients with an acute rejection a transient increase in the calculated fractional excretion of sIL-2Rα was seen while the IL-2Rα on lymphocytes was blocked. A decreased tubular reabsorption of sIL-2Rα as a consequence of interstitial damage is an attractive explanation (16). Anyhow, the calculated fractional excretion of sIL-2Rα seems not reliable to determine IL-2Rα blockade during episodes of acute rejection. The knowledge on the IL-2Rα status at that time however seems less relevant, while acute rejection episodes are treated with intensification of the immunosuppressive therapy anyway. No increase in the (fractional) excretion of sIL-2Rα was found in patients experiencing a CMV infection, tacrolimus toxicity or acute tubular necrosis. However the number of patients was too low to allow firm conclusions on this subject.

The clinical value of monitoring the level and duration of IL-2Rα blockade has still to be determined. While therapeutic drug monitoring is not necessary in patients treated with a standard dose of anti-IL-2Rα mAb, monitoring might be of value for several reasons. The duration of IL-2Rα blockade after a standard dose of basiliximab or daclizumab varies considerably (±4-6 weeks for basiliximab (2x 20 mg) versus ±16 weeks for daclizumab (5x 1mg/kg/week)), while both seem equipotent for the prevention of acute rejection (17). We found an unexpectedly long duration of IL-2Rα blockade after two doses of daclizumab (1 mg/kg) in tacrolimus and MMF treated recipients of a renal transplant (18). Besides population characteristics, the concomitant immunosuppressive medication might explain the variation in duration of IL-2Rα blockade in these studies (19). Therapeutic drug monitoring might be helpful to determine the minimum dose of the (expensive) IL-2Rα blocking mAb

required for effective rejection prophylaxis. Furthermore, monitoring might become relevant if alternative treatment protocols using anti-IL-2Rα mAbs are developed i.e. prolonged treatment in patients with severe toxicity of standard immunosuppressive drugs.

For clinical practice it is important to ensure activity of the agent administered to the patient. Therefore we have chosen a cut-off value for the calculated fractional excretion of 0.5 with a 100 percent specificity and sensitivity of 75 percent, and a cut-off value for the urinary sIL-2Rα concentration of 14 U/mmol creatinine with a similar specificity but lower sensitivity. Using these cut-off values, no patients with recurrence of IL-2Rα^{pos} lymphocytes in the circulation were missed. The consequence of a calculated fractional excretion above the proposed cut-off values depends on the immunosuppressive protocol. If the protocol requires extension of the duration of IL-2Rα blockade, either administration of daclizumab or flow cytometric analysis of IL2Rα^{pos} lymphocytes is advised. However, one has to consider that the gold standard (flow cytometric analysis) is not as robust as desired. The cut-off value to determine complete IL-2Rα blockade was arbitrarily set at the presence of less than 5% of CD25^{pos} lymphocytes in the CD3^{pos} population. So with flow cytometry a low level of IL-2Rα expression on lymphocytes was missed. The ELISA already measured sIL-2Rα in the urine in the absence of IL-2Rα^{pos} lymphocytes. So the ELISA seems a more sensitive method to detect the alpha chain of the IL-2 receptor than flow cytometry. Of course, the exact value of determining sIL-2Rα excretion can only be assessed in large clinical studies relating excretion of sIL-2Rα with clinical outcome.

In conclusion, blockade of the IL-2Rα after treatment with daclizumab can reliably be assessed by measuring the urinary concentration of sIL-2Rα or the calculated fractional excretion of sIL-2Rα. These findings can probably be extrapolated to other mAbs against the IL-2Rα. For clinical practice this method is easier to use and more sensitive than the flow cytometric analysis of IL-2Rα^{pos} lymphocytes. Monitoring of the IL-2Rα status of lymphocytes after treatment with daclizumab might be useful for guiding treatment. The cut-off values reported need to be confirmed in a prospective study.

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Chapter 6

Induction therapy with IL-2 receptor α blockade can replace corticosteroid therapy after renal transplantation. A prospective randomized multicenter study.

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Abstract

Steroids have been included in most immunosuppressive regimens after renal transplantation, but are feared for their side effects. Monoclonal antibodies against the alpha chain of the IL-2 receptor might give the opportunity to transplant steroid-free. We conducted a prospective, randomized, multicenter study to investigate whether this is feasible. A total of 364 patients were randomized to receive either daclizumab (1 mg/kg on days 0 and 10 after transplantation; steroid-free group $N = 186$), or steroids (0.3 mg/kg/day tapered to 0 mg at week 16 after transplantation; control group $N = 178$). All patients received tacrolimus, mycophenolate mofetil, and for the first three days 100 mg prednisolone intravenously. The primary endpoint was the incidence of biopsy-confirmed acute rejection within six months after transplantation. Biopsy-confirmed acute rejection was diagnosed in 27 patients in the steroid-free group (15%) and in 21 patients in the control group (12%) (95% confidence interval of difference: -4 to +10%, NS). Graft survival at 12 months was comparable in the two groups (steroid-free group: 91%; control group: 90 %). Mean arterial blood pressure, serum lipids, and incidence of patients with hyperglycemia were lower in the steroid-free group compared with controls. The immunosuppressive regimen of the steroid-free group was associated with increased costs. In conclusion, with the use of anti-IL-2Rα induction therapy it is feasible to avoid maintenance therapy with steroids in renal transplant recipients on tacrolimus and mycophenolate mofetil.

Introduction

Since the early sixties, corticosteroids have been the mainstay for acute rejection prophylaxis after organ transplantation. The use of corticosteroids is however associated with several well-documented side effects as hypertension, hyperlipidemia, new-onset diabetes mellitus, infections, accelerated bone loss, weight gain, and cosmetic changes (1). The availability of new, potent, and more selective immunosuppressive drugs has challenged the necessity of adding corticosteroids for rejection prophylaxis. The introduction of the calcineurin inhibitor cyclosporine was a major advance in organ transplantation, positively influencing graft survival. Studies concerning withdrawal of steroids in patients on cyclosporine based immunosuppressive therapy have shown an improvement in steroid-related side effects (2-5), but at the price of an increased incidence in acute rejection (6). Depending on the selection of the patients and timing of steroid-withdrawal, up to 50% of the patients had to resume steroids (7;8). The calcineurin inhibitor tacrolimus has been shown to be more potent than cyclosporine in preventing rejection (9;10). Experience with withdrawal of steroids in patients using a tacrolimus based immunosuppressive regimen is limited but promising, although steroid-withdrawal related rejection also occurred (11-13).

Daclizumab is a humanized monoclonal antibody directed against the alpha subunit of the IL-2 receptor (IL-2Rα-chain) and has been proven effective for rejection prophylaxis after transplantation (14;15). Daclizumab is well tolerated, and remarkably, the incidence of adverse events is not increased in patients treated with daclizumab. In pilot studies the addition of an IL-2Rα blocking antibody to a calcineurin inhibitor combined with mycophenolate mofetil, allowed avoidance of corticosteroids (16;17). So far, no randomized studies have been published comparing avoidance of steroids with steroid-withdrawal in patients on a tacrolimus based immunosuppressive regimen. In a prospective randomized controlled multi-center study we tested the hypothesis that the addition of daclizumab to tacrolimus and mycophenolate mofetil would preclude the need for corticosteroids without affecting the incidence of acute rejection. In the control group prednisone was tapered to zero in four months after renal transplantation as the best available alternative to limit the side effects of corticosteroids.

Materials and Methods

Study design

We performed a randomized trial at four kidney transplantation centers in the Netherlands. Patients were assigned either to treatment with daclizumab (steroid-free group) or to maintenance treatment with prednisone during the first four months after transplantation (controls). Adult recipients of a first or second transplantation from a living related or cadaveric donor were eligible for this study. Excluded were recipients of a graft from an HLA-identical living donor, patients taking prednisone or other immunosuppressive medications at the time of transplantation, patients with hemolytic uremic syndrome as original renal disease, premenopausal women not using adequate contraception, and patients with leukocytopenia or thrombocytopenia.

Shortly before transplantation, patients were randomly assigned to one of the treatment groups in an 1:1 ratio, with stratification for cadaveric heart-beating donor/ non-heart-beating donor/ living donor, and for center. Randomization was carried out by opening a sealed opaque envelope with the lowest available study number at each participating center. Both clinicians and patients were aware of the randomized assignment. The protocol was approved by the institutional review boards of the four participating hospitals, and written informed consent was obtained from all participants before transplantation.

Endpoints

The primary endpoint was the incidence of a first biopsy-proven acute rejection (Banff type 1 or higher) within six months after transplantation. Other endpoints included patient and graft survival, renal function, incidence of infections, wound dehiscence requiring surgical repair, incidence of new-onset diabetes mellitus during the first 12 months after transplantation, and blood pressure and lipid values at 2 months and 12 months after transplantation. The change in bone mineral density in the first 12 months after transplantation is reported separately (18).

Immunosuppression

In the steroid-free group, daclizumab (1 mg/kg body weight) was administered intravenously one hour before transplantation and once again on day 10-14 after transplantation. We aimed to block the IL-2Rα-chain for a period of at least 10 weeks after transplantation. Flow-

cytometric analysis of IL-2Rα^{pos} lymphocytes as described previously (19), was performed biweekly in whole blood. In case of reappearance of IL-2Rα^{pos} lymphocytes (as defined by a rise of the CD3^{pos}CD25^{pos} lymphocytes above 4% of the total lymphocyte population) within the first 10 weeks after transplantation, an additional dose of daclizumab was administered. The control population received prednisone 0.3 mg/kg/day orally for the first two weeks, and thereafter, the prednisone dose was gradually tapered to zero in four months.
Concomitant therapy was identical in the two groups. All patients received 100 mg prednisolone intravenously during the first three days after transplantation. Induction therapy with antilymphocyte therapy was not used. Tacrolimus was started on day one or two after transplantation at 0.15 mg/kg twice daily (orally) and the dose was subsequently adjusted to achieve target whole-blood trough concentrations of 15-20 ng/ml from day 0-14, 10-15 ng/ml from week 3-6, and 5-10 ng/ml after week 7. Tacrolimus concentrations in whole blood were measured by the IMx analyzer (Abbott Laboratories, Abbott Park, IL, USA). Mycophenolate mofetil (MMF) was administered at 1000 mg twice daily, and decreased to 750 mg twice daily at 2 weeks after transplantation, unless the patient weighed more than 90 kg.
Rejection episodes were primarily treated with methylprednisolone 500-1000 mg intravenously for three consecutive days. In case of a steroid-resistant rejection, antilymphocyte therapy was given (either rabbit polyclonal antithymocyte globulin or a mouse anti-CD3 monoclonal antibody). In case of interruption of tacrolimus or a reduction of the MMF dose below 1000 mg/day, or treatment with antilymphocyte therapy, administration of prednisone (dose 0.10-0.15 mg/kg/day) was allowed.

Additional medication

All patients received prophylaxis for peptic ulcers, and *Pneumocystis Carinii Pneumonia* (cotrimoxazole 480 mg daily). Suppletion of elementary calcium (500 mg) and CMV prophylaxis was given according to center praxis.

Assessments

Data on rejection episodes, dialysis requirements, concomitant medication, adverse events, and infections were recorded throughout the entire study period. A biopsy was performed in cases of deteriorating graft function without an obvious pre- or postrenal cause. No protocol biopsies were performed. Biopsies were examined by the local nephropathologist and were

classified according to the Banff 1997 Biopsy scoring system (20). For patients who underwent several biopsies during a rejection episode, the one with the most severe histologic score was recorded. Presumed rejection was defined as treatment with methylprednisolone without performing a graft biopsy, or treatment initiated with antirejection therapy while the biopsy showed only borderline rejection or no signs of rejection. For calculation of the creatinine clearance, the Cockcroft-Gault formula was used (21). Infections were defined as any treatment episode with antimicrobial drugs except for the use of prophylaxis. Infections were classified using the Centers for Disease Control and Prevention's definitions for nosocomial infections (22).

Statistical Analyses

Results are given as means ± SD unless stated otherwise. The statistical analyses were performed on an intention-to-treat basis. In the primary analysis *P*-values and 95% confidence intervals were calculated using exact methods for the difference of proportions and t-tests for the difference of continuous variables. Time to first biopsy confirmed episode of rejection is given as Kaplan-Meier curves and compared using the log-rank test. For this analysis, data were censored at the time of graft failure due to non-immunological causes, at the time of death, at the time of loss to follow-up, or at the end of follow-up. For all tests a *P*-value less than 0.05 (two-sided) was considered significant.

Results

Between October 1999 and March 2002, 381 patients were enrolled into the study. A total of 192 patients were assigned to the steroid-free group, and 189 to the control group. Eleven patients randomized to the control group and six patients randomized to the steroid-free group were excluded from analysis for several reasons, leaving 364 patients for intention-to-treat analysis (Figure 1). Two patients in the steroid-free group and one patient in the control group were lost to follow-up at six months after transplantation and were excluded from the analysis of the endpoints measured at twelve months. The proportion of male patients was higher in the steroid-free group compared with the control group. The two groups were not different with respect to the other baseline characteristics as shown in Table 1. In the steroid-free group 28 patients (15%) either had a PRA above 50% or were recipients of a second renal transplant as compared to 31 patients in the control group (17%).

Table 1. Baseline characteristics of patients and donors.

Characteristic	Steroid-free *N* = 186	Control *N* = 178
Median (range) age patients (years)	48 (18-78)	49 (19-73)
Number of male patients (%)	133 (72) [a]	102 (57)
Cause of end-stage renal disease (%)		
Glomerulonephritis	27	24
Chronic pyelonephritis	13	12
Polycystic kidney disease	15	19
Diabetes mellitus	9	7
Renovascular disease	3	0
Other	15	19
Unknown	18	20
Hemodialysis/peritoneal dialysis /no dialysis	94/69/23	89/73/16
Median (range) duration of dialysis (months) [b]	34 (1-250)	32 (1-143)
Percentage of group with PRA = 0 to 10% /11 to 49% /50 to 100%	80/10/10	74/16/10
First/second graft (n)	167/19	157/21
Number of patients (%) with diabetes mellitus before transplantation	23 (12%)	14 (8%)
Number of patients requiring insulin	21	13
Cadaveric heart-beating/ non-heart-beating/ living related/ living unrelated donor (n)	72/45/43/26	75/40/37/26
Median (range) cadaveric donor age (years)	49 (2-75)	49 (14-75)
Median (range) living donor age (years)	52 (24-77)	50 (21-75)
Mean HLA-antigen mismatches (n)	2.5±1.3	2.3±1.3
Mean (SD) cold ischemia time (h) [c]	20.4±7.6	20.6±6.8

[a] $P<0.05$ between groups.

[b] Excluding patients transplanted before renal replacement therapy was started.

[c] Cadaveric kidneys only.

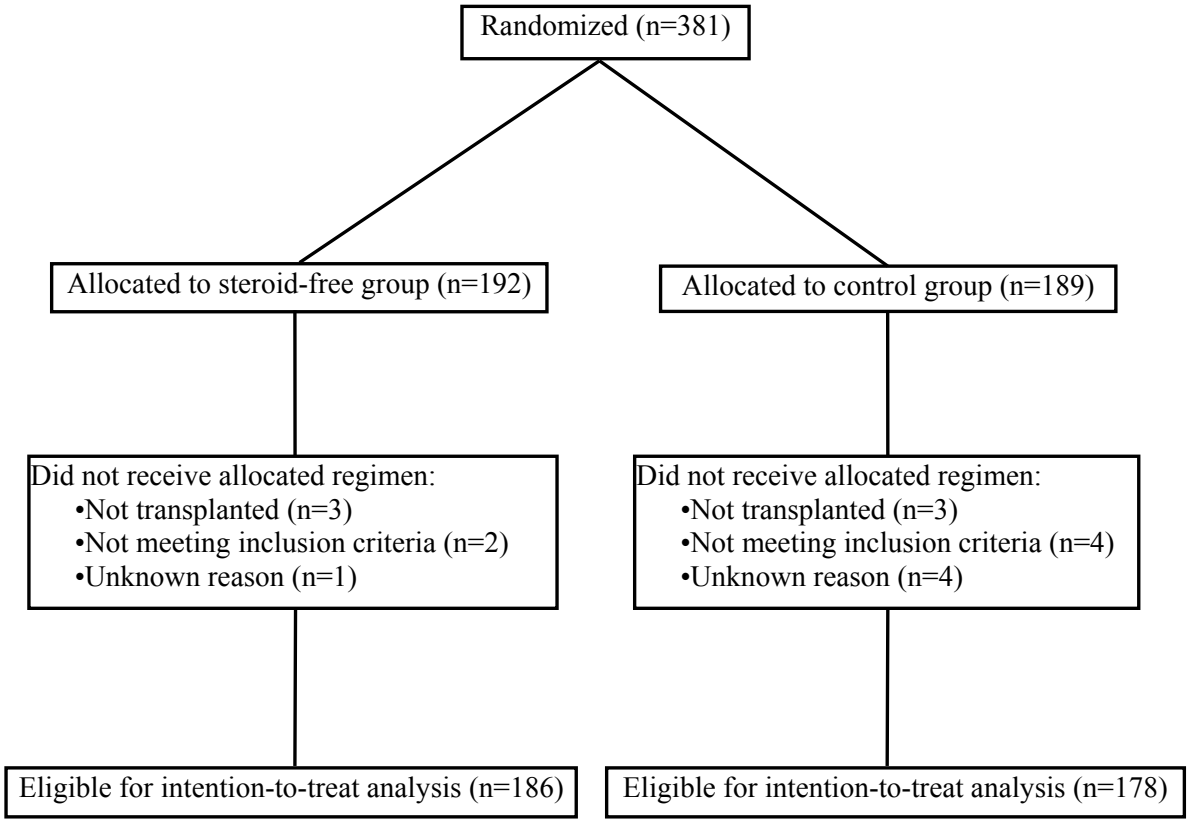

Figure 1. Flow diagram of the patients randomized to one of the treatment groups.

Protocol adherence

Three of 186 patients (2%) received an extra dose of daclizumab because of recurrence of IL-2Rα^{pos} lymphocytes in the peripheral circulation within 10 weeks after transplantation. In 55 of 186 patients (28%) in the steroid-free group initiation of prednisone was judged necessary for clinical reasons (42 patients: interruption of other immunosuppressive drug(s), 10 patients: rejection, 3 patients: miscellaneous). At twelve months after transplantation 31 of 186 patients (17%) in the steroid-free group and 28 of 178 patients (16%) in the control group were still treated with prednisone. The median cumulative steroid dose at 12 months after transplantation (excluding pulse therapy) was 300 mg per patient (range 300-6800 mg) in the steroid-free group and 1607 mg per patient (range 585-5343 mg) in the control group (P<0.001).

Cessation or interruption for more than 14 days of one or more of the immunosuppressive drugs was judged necessary for clinical reasons in 39 patients in each group. In the steroid-free and control group respectively, tacrolimus was discontinued for more than 14 days in 11 and 10 patients, MMF in 22 and 26 patients, and tacrolimus together with MMF in 6 and 3

patients. The reasons for discontinuing of tacrolimus in the steroid-free group and controls were nephrotoxicity (6 versus 1), neurotoxicity (1 versus 3), pain of the bones (2 versus 2), diabetes mellitus (0 versus 3), and other reasons (8 versus 4). The reasons for discontinuing of MMF were gastrointestinal intolerance (12 versus 10), CMV infection (10 versus 6), leukocytopenia (3 versus 8), and other reasons (3 versus 5).
In the steroid-free group tacrolimus trough levels were slightly lower compared with the controls at month two (10.2±2.8 versus 11.2±3.9 ng/mL, $P<0.01$) and six (7.7±2.3 versus 8.6±2.6 ng/mL, $P<0.01$) after transplantation. At all other time points there were no differences between the two groups in tacrolimus trough levels.

(Biopsy-proven) acute rejection episodes
The incidence of biopsy-proven acute rejection within the first six months was 15% in the steroid-free group and 12% in the control group (NS) (Table 2). The severity of the rejection episodes was comparable in the two groups. A type III rejection was observed only once in a patient in the control group. All the other acute rejection episodes in the total population were classified as type I or II. The time interval to the first biopsy-proven acute rejection was comparable in the steroid-free group and the controls: median 11 versus 14 days (range 4-157 days versus 1-127 days, NS) (Figure 2). No difference between the groups was found in the proportion of patients having a presumed rejection, a second biopsy-proven rejection, and the proportion of patients treated with antilymphocyte therapy. A biopsy excluded the presence of an acute rejection in 13 patients with a presumed rejection in the steroid-free group and in 10 patients in the control group, and in 4 and 2 patients respectively, a borderline acute rejection was found. In the subgroup of patients with either a PRA above 50%, or a second renal transplant, 8 of 28 patients (29%) in the steroid-free group experienced a biopsy-proven acute rejection as compared to 4 of 31 patients (13%) in the control group (NS). Multivariate analysis including the covariates treatment assignment, center, type of the donor (cadaveric heart-beating; non-heart-beating; living), and gender did not change the results.

Patient and graft survival
At twelve months after transplantation, patient and graft survival were not different between the two groups (Table 3). Causes of patient death in the steroid-free group and the controls included cardiovascular events (7 versus 3), infections (1 versus 4), lymphoma (1 versus 0) and other reasons (0 versus 3). Causes of graft loss were rejection (4 versus 1), vascular thrombosis (7 versus 8), primary non-function (4 versus 4), hemolytic uremic syndrome (1 in steroid-free group), and infection, bleeding and other causes (2, 1, and 3 in control group).

Table 2. Rejection episodes during the first 6 months after transplantation.

	Steroid-free *N* = 186	Control *N* = 178	Difference (%) (95% CI) [a]
Biopsy-proven acute rejection (%)	27 (15)	21 (12)	+3 (-4 to +10)
Type of biopsy-proven acute rejection			
Type I (%)	9 (5)	8 (4)	0 (-4 to +5)
Type II or III (%)	18 (10)	13 (7)	+3 (-3 to +8)
Biopsy-proven or presumed rejection (%)	54 (29)	38 (21)	+8 (-1 to +17)
Second (biopsy-proven) rejection [b] (%)	6 (3)	1 (1)	+3 (-0 to +5)
Episode requiring antilymphocyte therapy (%)	11 (6)	4 (2)	+4 (-0 to +8)

[a] 95% confidence interval of the difference.

[b] If a patient had multiple rejection episodes and the episodes occurred within a time interval of 14 days, the episodes were collapsed and considered as a single event.

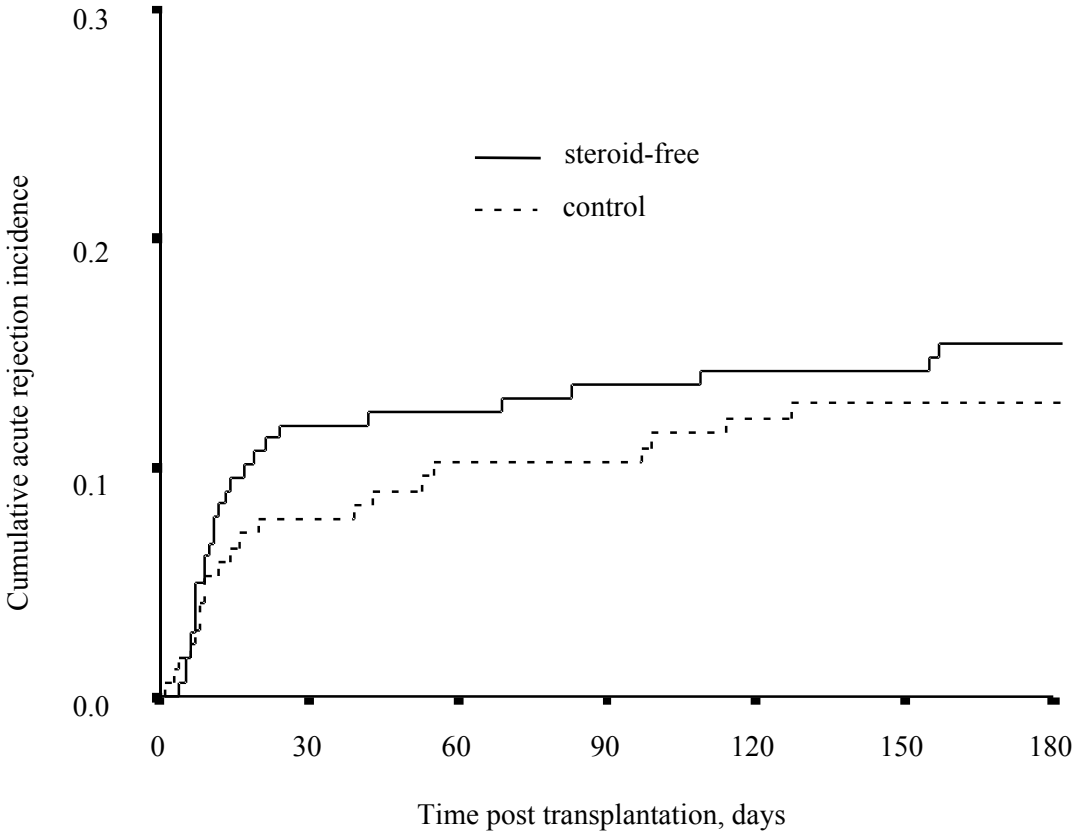

Figure 2. Kaplan-Meier estimate of the cumulative probability of a biopsy-proven acute rejection episode during the first six months after transplantation (steroid-free versus controls: P=0.48).

Table 3. Patient and graft survival, incidence of infections, wound dehiscence requiring surgical repair, and new-onset diabetes mellitus at 12 months after transplantation.

	Steroid-free *N* = 184	Control *N* = 177	Difference (%) (95% CI) [a]
Patient survival (%)	175 (95)	167 (94)	+1 (-4 to +5)
Graft survival (%)	168 (91)	159 (90)	+2 (-5 to +8)
Patients with at least one infection (%)	118 (64)	105 (59)	+5 (-5 to +15)
Patients with wound dehiscence requiring surgical repair (%)	0 (0)	5 (3)	-3 (-5 to -0) [b]
Patients with new-onset diabetes mellitus (%)	12 (7)	21 (12)	-5 (-11 to +1)

[a] 95% confidence interval of the difference.

[b] $P<0.05$

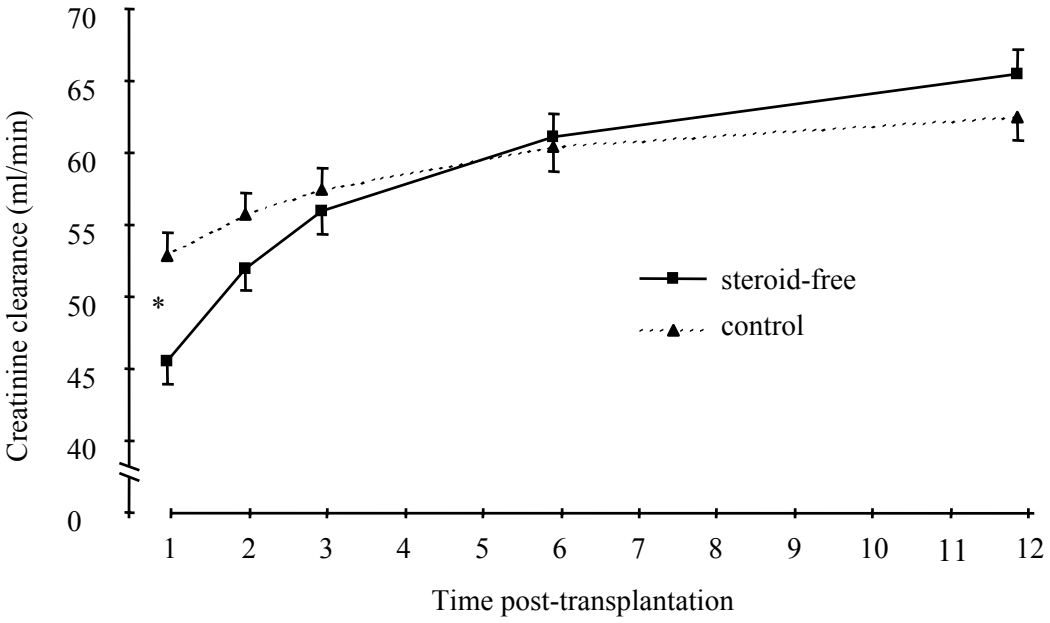

Figure 3. Mean (±SE) calculated creatinine clearance (*: $P< 0.01$ between groups).

Graft function

The incidence of delayed graft function, defined as the need for dialysis after transplantation was similar in both groups (24% versus 22%, NS). Figure 3 shows that the calculated creatinine clearance was slightly lower in the steroid-free group at one month after transplantation (45±20 ml/min versus 53±21 ml/min, $P<0.01$) while no differences were found later on (t=12 months: 65±21 ml/min versus 62±21 ml/min). Excluding patients with rejection or overt tacrolimus toxicity revealed similar results. The number of patients with proteinuria (>1 g/day) was comparable in both groups at 12 months after transplantation (10 versus 14 patients, NS).

Episodes of graft dysfunction that resulted in performing a biopsy were more frequent in the steroid-free group compared with controls (number of biopsies: 124 versus 85). In 85 of 186 patients (46%) in the steroid-free group and in 63 of 178 patients (35%) in the control group a biopsy was performed ($P<0.05$).

Incidence of infections, malignancies, and wound dehiscence

There was no difference between the groups in the proportion of patients that experienced at least one infection (Table 3). Also, the number of infections per patient was comparable in both groups (1.2±1.4 versus 1.4±1.5 per patient, NS).

Twelve malignancies were recorded during the 12-month assessment period, six in the steroid-free group (carcinomas of bronchus, prostate, skin, mamma, colon, and bladder), and two in the control group (carcinomas of prostate and skin). Two patients in each group had a post-transplant lymphoproliferative disorder.

Wound dehiscence requiring surgical repair only occurred in five patients in the control group early after transplantation ($P<0.05$) (Table 3).

New-onset diabetes mellitus, blood pressure and lipids

During the first 12 months after transplantation new-onset diabetes mellitus, defined as the institution of treatment with glucose lowering medication, developed in 12 of 186 patients (7%) in the steroid-free group and in 21 of 178 patients (12%) in the control group (NS; Table 3). Diabetes persisted until the end of follow-up in 11 and 17 patients respectively. Three patients, all assigned to the control group, had a transient increase in the serum concentration of glucose above 11.1 mmol/L. Although they met the criteria for diagnosing diabetes (23), they were never treated with glucose lowering medication. Including the latter patients in the

analysis revealed a significantly lower risk to develop diabetes mellitus in the steroid-free group (difference with controls: -7% (95% CI: -13 to -1, $P<0.05$)).
At 2 months after transplantation mean arterial blood pressure was significantly lower (4 mmHg, $P<0.01$) in the steroid-free group compared with the controls, while an equal amount of antihypertensive drugs was used (Table 4). At twelve months there was no difference between the two groups in mean arterial blood pressure. At two months after transplantation mean total cholesterol, high-density lipoprotein cholesterol, and triglycerides were significantly lower in the steroid-free group compared with controls (Table 4). At twelve months after transplantation these differences had disappeared. A comparable number of patients were treated with cholesterol lowering medication at this moment (16 versus 13 patients, NS).

Costs

On average 157±40 mg of daclizumab was administered to patients allocated to the steroid-free group. Based on an actual price of 320 Euro's/25 mg of daclizumab, the cost of daclizumab amounted ±2000 Euro's per patient in the steroid-free group. The control group used a median 1300 mg of prednisone in excess of the steroid-free group. Based on an actual price of 0.09 Euro's/10 mg prednisone, the additional cost of prednisone amounted ±12 Euro's per patient in the control group.

Table 4. Blood pressure and lipids at 2 and 12 months after transplantation. [a]

Parameter	At 2 months after transplantation			At 12 months after transplantation		
	Steroid-free	Control	Difference (%) (95% CI) [b]	Steroid-free	Control	Difference (%) (95% CI) [b]
Mean arterial pressure (mmHg)	97±12	101±11	-4 (-6 to -1) [c]	98±12	99±11	-1 (-4 to +2)
Number of antihypertensive drugs	0.9±0.7	1.1±0.9	-0.2 (-0.3 to +0.0)	1.1±0.9	1.1±1.0	+0.0 (-0.2 to +0.2)
Total cholesterol (mmol/L)	4.9±1.2	5.4±1.5	-0.5 (-0.8 to -0.2) [c]	5.2±1.3	5.3±1.1	-0.1 (-0.4 to +0.2)
HDL cholesterol (mmol/L)	1.02±0.31	1.23±0.46	-0.2 (-0.3 to -0.1) [c]	1.11±0.34	1.21±0.45	-0.1 (-0.2 to +0.0)
LDL cholesterol (mmol/L)	2.99±0.95	3.11±1.09	-0.1 (-0.4 to +0.1)	3.12±1.03	3.16±0.93	-0.0 (-0.3 to +0.2)
Triglycerides (mmol/L)	2.0±1.1	2.4±1.6	-0.4 (-0.7 to -0.1) [d]	2.1±1.5	2.0±1.6	+0.0 (-0.3 to +0.5)
Ratio cholesterol /HDL cholesterol	5.3±2.0	5.0±2.3	+0.3 (-0.2 to +0.8)	5.0±2.1	4.9±2.1	+0.2 (-0.4 to +0.7)

[a] Analysis was confined to patients alive with a functioning graft at 12 months after transplantation.

[b] 95% confidence interval of the difference.

[c] $P<0.01$.

[d] $P<0.05$.

Discussion

In recipients of a renal transplant who were treated with tacrolimus, MMF, and three days of prednisolone, induction therapy with daclizumab without additional steroids resulted in a comparable incidence of biopsy-proven acute rejection as continuation of steroids until four months after transplantation. This was found despite a clear difference in the cumulative steroid dose between the treatment groups. So far, this is the first large prospective randomized trial comparing limited exposure to steroids with avoidance of steroids after renal transplantation. It clearly shows that daclizumab induction therapy allows renal transplantation to be performed successfully with only three days of postoperative corticosteroid therapy. More than 80% of the patients in both groups were free from steroid treatment at 12 months after transplantation, which is an impressive success rate compared with studies concerning steroid-withdrawal in patients on cyclosporine based immunosuppressive regimens (7;8). Recipients of a second renal transplant or patients with a high PRA accounted for more than 15% of the study population, so the study was not restricted to low-risk recipients only. The incidence of biopsy-proven acute rejection in the control population (12%) was similar to preliminary reports in patients on tacrolimus and MMF based immunosuppression with limited exposure to prednisone (11;24). In both treatment arms the majority of the rejection episodes were steroid responsive. Only 15 patients in the total population (4%) were treated with antilymphocyte therapy for a steroid-resistant acute rejection, which compares well with large studies on immunosuppression with the combination of tacrolimus, MMF and corticosteroids (12;25). A tendency to an increased need for antilymphocyte therapy and to an increased incidence in second biopsy-proven acute rejection was found in the steroid-free group. The relevance of this finding is unclear. Both regimens resulted in similar patient and graft survival rates. At the end of follow-up there was no difference in graft function between the two groups. At one month after transplantation, graft function was slightly better in patients treated with corticosteroids. This finding was independent of the occurrence of a rejection or overt calcineurin inhibitor toxicity as was also shown before (26;27), and is possibly explained by a direct hemodynamic effect of steroids on glomerular filtration (28).

Traditionally, daclizumab is administered in a five-dose regimen (1 mg/kg every 14 days) (14;15). The duration of IL-2Rα blockade required for optimal rejection prophylaxis has not

been determined yet, and in recent trials we and others have observed that fewer doses of daclizumab may be safe and effective after organ transplantation (29-32). In the present study only two doses of daclizumab were administered, and IL-2Rα blockade was maintained beyond 10 weeks after transplantation in the vast majority of the patients as was also reported earlier (30). In the steroid-free group, 23 of the 27 biopsy-proven acute rejection episodes (85%) occurred within the first 10 weeks after transplantation, during IL-2Rα blockade. We have shown previously that other cytokines can take over the role of IL-2 as T-cell growth factor during IL-2Rα blockade (33). Therefore, we expect no additional effect on acute rejection prevention by extending the duration of IL-2Rα blockade with additional doses of daclizumab.

Based on the design of the study, differences in steroid-related side effects could especially be expected during the first four months after transplantation. In the steroid-free group less patients developed diabetes mellitus compared with controls. However, consistent with other reports (3;34;35), diabetes mellitus was transient in a proportion of patients after withdrawal of steroids. Consequently, a similar percentage of patients remained diabetic in both groups.

At two months after transplantation mean arterial blood pressure was slightly lower in the steroid-free group. The total and HDL cholesterol concentrations were lower in the steroid-free group (on average 9% and 17% respectively). As a consequence, the ratio between total and HDL cholesterol, which is known to be a cardiovascular risk factor, did not differ between the groups. This finding is in accordance with previously described effects of steroids on lipid metabolism after renal transplantation (26;27;36). At twelve months after transplantation there were no differences between the two groups in serum lipids and mean arterial blood pressure. The meaning of these temporary reductions in the frequency of diabetes mellitus and in blood pressure is unclear, but it might be of benefit in those patients at high cardiovascular risk (37).

No difference between the groups was found in the incidence of infections, possibly due to the systematic use of antiviral and antimicrobial prophylaxis. Finally, there was a significantly increased incidence of wound dehiscence requiring surgical repair in the group with limited

steroid exposure. Although steroid use is related with wound dehiscence after surgery (38), the numbers in our study are small and these results should therefore be interpreted with caution.

A global cost analysis shows that the steroid-free regimen was more expensive than the regimen with limited steroid-exposure. Besides the increased need to perform a renal biopsy and the tendency to increased usage of ATG in the steroid-free group, induction therapy with two doses of daclizumab was more costly than four months corticosteroid therapy.

Inherent to its design our study also has some limitations. The so-called steroid-free regimen was not completely steroid-free as patients received prednisolone for the first three days after transplantation. The influence of three days of 100 mg prednisolone intravenously on parameters such as glucose metabolism and infections is unknown. Furthermore we have not proven that treatment with daclizumab is really necessary to achieve the results that were obtained in our steroid-free group. This would have required an additional study group, only treated with tacrolimus and MMF.

In conclusion, treatment with two doses of daclizumab enabled a steroid-free immunosuppressive regimen of tacrolimus and mycophenolate mofetil in recipients of a renal transplant. There was no difference in the incidence of biopsy-proven acute rejection compared with a regimen with limited steroid exposure. Our results indicate a beneficial effect on the cardiovascular risk profile of renal transplant recipients treated with a steroid-free immunosuppressive regimen. This beneficial effect should be balanced against the increased costs associated with the steroid-free regimen.

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Chapter 7

Prevention of accelerated bone loss with a steroid-free immunosuppressive regimen and a regimen with four months of steroids in the first year after renal transplantation.

A prospective randomized multicenter study.

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Abstract

Steroid-related bone loss is a recognized complication after renal transplantation. In a prospective, randomized, multicenter study we compared the influence of a steroid-free immunosuppressive regimen to a regimen with limited steroid exposure on the changes in bone mass after renal transplantation. A total of 364 patients were randomized to receive either daclizumab (1 mg/kg on days 0 and 10 after transplantation; steroid-free group n=186), or prednisone (0.3 mg/kg/day tapered to 0 mg at week 16 after transplantation; controls n=178). All patients received tacrolimus, mycophenolate mofetil, and during the first three days 100 mg prednisolone intravenously. Changes in bone mineral density (BMD) were evaluated in 135 patients in the steroid-free group and in 126 controls. The mean (±SD) BMD of the lumbar spine decreased slightly in both groups during the first 3 months after transplantation (steroid-free: -1.3±4.0% (P<0.01); control group: -2.3±4.2% (P<0.01)). In the following months lumbar BMD recovered in both groups (P<0.01), resulting in a lumbar BMD at 12 months after transplantation comparable to the baseline value. No difference between the groups was found at three months (steroid-free versus controls: +1.0%; 95% confidence interval: -0.0% to +2.0%, NS) and at twelve months after transplantation (steroid-free versus controls: +0.9%; 95% confidence interval: -0.8% to +2.6%, NS). Similar results were obtained for the changes in BMD of the femoral neck in both groups. In conclusion, the steroid-free regimen results in a comparable preservation of bone mass as the regimen with limited steroid exposure.

Introduction

Renal transplant recipients are at increased risk to develop bone fractures compared with the healthy population (1). The fracture risk is especially increased early after transplantation (2). Low bone mineral density (BMD) has also been shown a risk factor for fractures in renal transplant recipients (3-5). During the first six months after transplantation BMD has been shown to decrease between 2 and 7% percent in cyclosporine and prednisone treated patients (6-11). Hereafter the decrease in BMD seemed to approach the normal age-related level (6;12;13). Therefore, prevention of bone loss has its greatest impact in the first period after transplantation. Histomorphometric analysis of bone biopsies (8) and several cross-sectional studies (12;14;15) indicate a causal relationship between bone loss and the use of glucocorticoids in renal transplant recipients. Avoidance of steroids would therefore be an attractive option, and several studies suggest that bone loss after transplantation is prevented with a steroid-free immunosuppressive regimen (16;17). These studies however were performed in a highly selected population (16) or without controls (17). Therefore we performed a prospective, randomized, multicenter study to investigate whether a steroid-free immunosuppressive regimen prevents accelerated bone loss compared with a regimen with limited steroid exposure. In the control group prednisone was tapered to zero in four months as the best available alternative to limit accelerated bone loss after transplantation. In another article we have described that both regimens resulted in a comparable incidence of biopsy-proven acute rejection of 12 to 15% (18). The steroid-free regimen was associated with a temporary beneficial effect on the cardiovascular risk profile of the renal transplant recipients, but also with increased costs.

Materials and Methods

Study design

We performed a randomized trial at four kidney transplantation centers in the Netherlands. Patients were assigned either to treatment with daclizumab (steroid-free group) or to maintenance treatment with prednisone during the first four months after transplantation (controls). Adult recipients of a first or second transplantation from a living related or cadaveric donor were eligible for this study. In another paper we have described the exclusion criteria, the details of randomization, and the immunosuppressive protocol (18).
All patients received tacrolimus and mycophenolate mofetil (MMF) as maintenance therapy, and prednisolone 100 mg/day intravenously during the first three days after transplantation. The steroid-free group received the monoclonal antibody daclizumab (1 mg/kg body weight) intravenously one hour before transplantation and once again on day 10-14 after transplantation. The control group received prednisone 0.3 mg/kg/day orally for the first two weeks and thereafter the prednisone dose was gradually tapered to zero in 16 weeks. Acute rejection was primarily treated with methylprednisolone 500-1000 mg intravenously for three consecutive days. In case of interruption of tacrolimus or a reduction of the MMF dose below 1000 mg/day, or treatment with antilymphocyte therapy, administration of prednisone (dose 0.10-0.15 mg/kg/day) was allowed. Treatment with elementary calcium (500 mg/day) was given according to center praxis.

End points

The end points were the percent change in BMD of the lumbar spine and the femoral neck at three and twelve months after transplantation.

Bone densitometry

Bone mineral density was assessed at two weeks after transplantation (baseline value) with subsequent measurements being performed at 3 and 12 months. Bone mineral density (BMD) of the lumbar spine (L2-L4 anteroposterior direction) and the femoral neck were measured with dual energy X-ray absorptiometry (DXA), using the Hologic QDR 4500 densitometer (Hologic Inc., Waltham, MA, USA) in two centers and the Lunar DPX densitometer (Lunar Radiation Corp., Los Angeles, CA, USA) in the other two centers. All bone mineral density measurements in one patient were performed using the same densitometer. Z scores were calculated using the reference database of the densitometer utilized. The coefficient of variation (CV) of daily measurements of the lumbar spine phantom was 0.4% to 1.0% in the

four centers. The Hologic spine phantom was scanned on each instrument ten times to establish instrument cross-calibration values (19). BMD values were transformed to standardized values and expressed in mg/cm^2 (20). Changes in BMD over the study period were expressed as percent change from the baseline value. The technicians who performed BMD measurements were not aware of the group assignment of the patients.

Radiography

A radiograph of the lumbar spine was obtained at baseline. If a vertebral fracture was diagnosed, the densitometry result of this vertebra was excluded from the analysis.

Biochemical measurements

Calcium, phosphorus, and alkaline phosphatase were measured at one month (baseline value), 3, and 12 months after transplantation by standard autoanalyzer methods. Intact parathyroid hormone (iPTH) was assessed before transplantation, and at 3 and 12 months after transplantation, using a commercial assay.

Calculations and statistical analyses

The serum total calcium concentration was adjusted for albumin following the equation: Adjusted calcium (mmol/L) = calcium – (0.025 * albumin) + 1 mmol/L (21). The creatinine clearance was estimated using the Cockcroft-Gault equation (22).

Results are given as means ± SD unless stated otherwise. The statistical analyses were performed on an intention-to-treat basis, including only patients in whom the changes from baseline in BMD could be assessed. In the primary analysis P-values and 95% confidence intervals were calculated using t-tests for the difference of continuous variables between the groups. Differences with baseline were assessed with paired t-tests. Log transformation was performed prior to the analysis where appropriate. Chi-square analysis was performed to compare proportions between the two groups. For all tests a P-value less than 0.05 (two-sided) was considered significant.

Results

Patients

Between October 1999 and March 2002, 381 patients were enrolled. Seventeen patients randomized did not receive the allocated regimen for several reasons, leaving 364 patients for intention-to-treat analysis (Figure 1). In the steroid-free group and control group 51 and 52 patients respectively failed to complete a baseline and at least one follow-up DXA measurement at the scheduled time. These patients were also excluded from analysis, leaving 135 patients in the steroid-free group and 126 patients in the control group (Figure 1). The BMD measurements were performed at similar time points in the steroid-free and control group (baseline: 14±5 versus 13±5 days after transplantation; t= 3 months: 97±11 versus 98±13 days after transplantation; t= 12 months: 369±22 versus 371±20 days after transplantation). The proportion of male patients was higher in the steroid-free group compared with the control group. The two groups were not different with respect to the other baseline characteristics, as shown in table 1. The baseline characteristics of the patients who were excluded from analysis were not different from those included in the analysis.

Immunosuppression and acute rejection episodes

In accordance with the study design, the control group received more prednisone, especially in the first three months after transplantation (Table 2). The number of patients treated with methylprednisolone pulse therapy because of a (presumed) rejection was comparable in both groups (Table 2). In 36 patients (27%) in the steroid-free group prednisone was started for several clinical reasons (intolerance for MMF (n= 13), intolerance for tacrolimus (n=16), the occurrence of a (severe) acute rejection (n=5), and other causes (n=2)). At twelve months after transplantation 21 patients (16%) in the steroid-free group and 16 patients (13%) in the control group were still using prednisone (NS). There was no difference between the groups in the proportion of patients treated with bisphosphonates, vitamin D, or patients with a parathyroidectomy in the first year after transplantation (Table 2). Bisphosphonates were administered because of (symptomatic) hypercalcemia (n=16), and for unknown reason (n=2). Vitamin D was administered because of a history of parathyroidectomy (n=4), treatment of hyperparathyroidism (n=2), vitamin D deficiency (n=1) and for unknown reasons (n=3).

Table 1. Baseline characteristics of patients.

Characteristic	Steroid-free N= 135	Control N= 126
Median (range) age patients (years)	50 (21-78)	49 (19-73)
Number of male patients (%)	103 (76) [a]	72 (57)
Number of pre-/ postmenopausal women	17/15	26/28
Body weight (kg)	75±14	73±15
Body mass index (kg/m^2)	25±4	25±5
Number of patients (%) with diabetes mellitus	15 (11)	9 (7)
Number of patients (%) with bone fractures [b]	6 (4)	6 (5)
Number of patients (%) with total parathyroidectomy	2 (2)	2 (2)
Hemodialysis/ peritoneal dialysis/ no dialysis	67/ 50/ 18	62/ 51/ 13
Median (range) duration of dialysis (months) [c]	32 (1-245)	30 (2-111)
First/second graft (n)	123/ 12	113/ 13
Bone mineral density of lumbar spine (mg/cm^2)	1118±188	1091±178
Z score [d]	-0.4±1.5	-0.4±1.4
Bone mineral density of femoral neck (mg/cm^2)	821±153	816±147
Z score [d]	-0.6±1.2	-0.6±1.2

[a] $P<0.05$ between groups.

[b] History of fracture or fracture at X-ray of the lumbar spine at baseline.

[c] Excluding patients transplanted before renal replacement therapy was started.

[d] SD of age-matched and gender-matched controls.

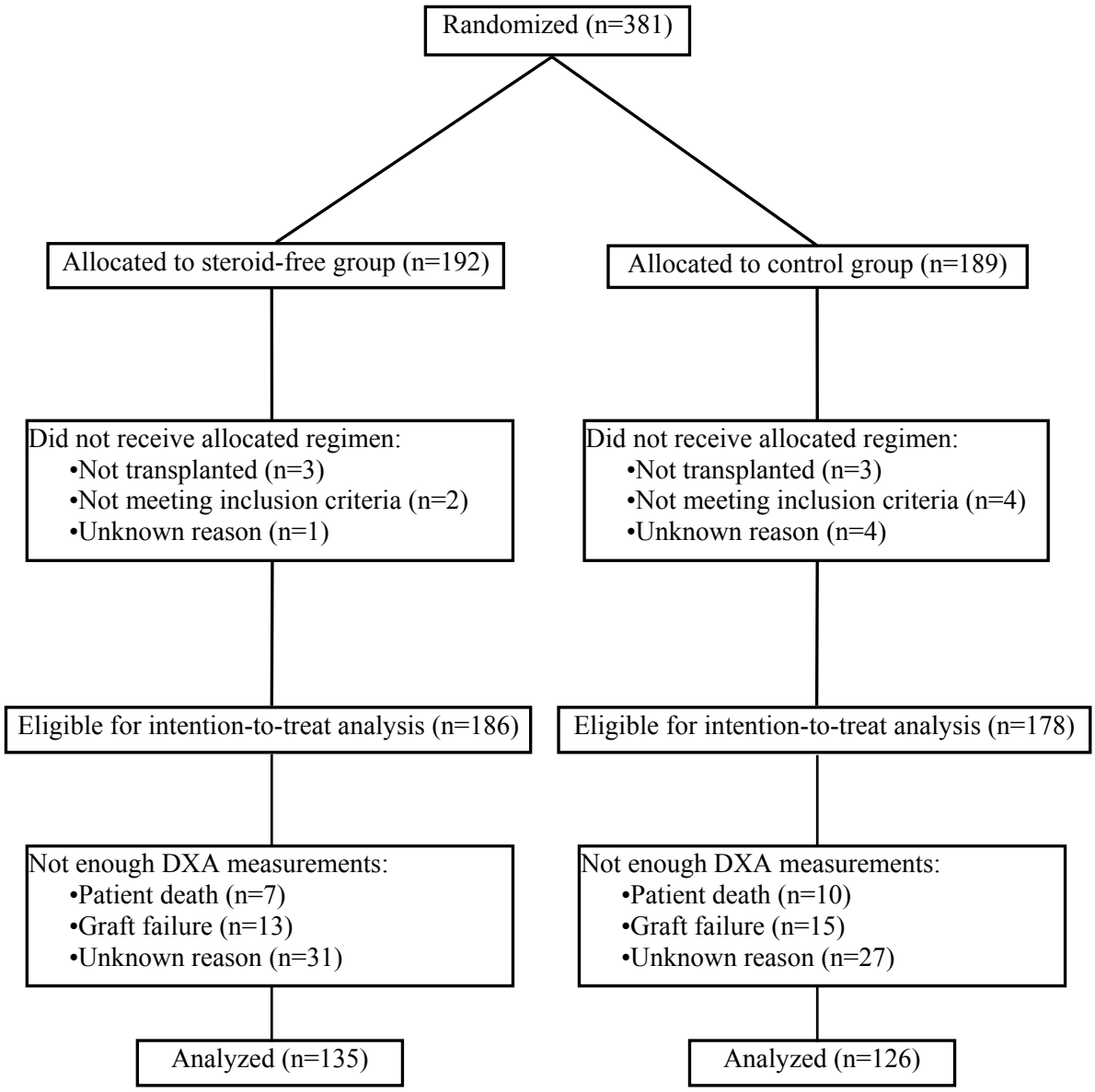

Figure 1. Flow diagram of the patients randomized to one of the treatment groups.

Bone mineral density measurements and fractures

At baseline, BMD was similar in the two groups at both skeletal sites (Table 1). At three months as well as at twelve months after transplantation, the percent change in BMD at any site was not different between both groups (Figure 2). In the first three months after transplantation, lumbar BMD decreased significantly ($P<0.01$) in the steroid-free (-1.3±4.0%) and in the control group (-2.3±4.2%) (difference between groups: +1.0% (95% CI: -0.0 to +2.0; NS)). During the following months there was a significant recovery of lumbar BMD in

both groups (P<0.01), resulting in a lumbar BMD at 12 months after transplantation comparable with baseline in both groups (difference between groups: +0.9% (95% CI: -0.8 to +2.6; NS)).
BMD of the femoral neck also decreased significantly (P<0.05) in both groups (-1.4±5.1%) during the first three months after transplantation (difference between groups: -0.0% (95% CI: -1.3 to +1.3; NS)). In the following months there was a non-significant recovery of femoral neck BMD in both groups, resulting in a BMD of the femoral neck at 12 months after transplantation which was comparable to the baseline value in the steroid-free group and control group (difference between groups: -0.1% (95% CI: -2.2 to +1.9; NS)). Adjustment for the imbalance in gender between the treatment groups by covariate analysis did not essentially change the results.
In the steroid-free group 70 patients (52%), were not treated with prednisone or methylprednisolone pulse therapy during follow-up. The changes in BMD of the lumbar spine and femoral neck in this subgroup were also not different from the changes in the control group. However, the percent of lumbar BMD lost at twelve months after transplantation in patients who were still using prednisone at twelve months after transplantation (-2.2±8.3%) was significantly (p<0.05) larger compared with the patients who did not use prednisone (+0.7±6.3%) (difference between groups: -2.9%; 95% CI: -0.4 to -5.4). During follow-up fractures of the ribs occurred in three patients (steroid-free versus control group: 2 versus 1).

Renal function

The creatinine clearance was comparable in the steroid-free and the control group at three and twelve months after transplantation (t=3 months: 55±19 versus 57±18 ml/min; t=12 months: 67±21 versus 62±20 ml/min). In the population analyzed, graft failure occurred in three patients in each group during follow-up.

Biochemical measurements

Biochemical data are summarized in Table 3. The serum concentrations of adjusted calcium, and phosphorus were significantly higher in the steroid-free group compared with the control group at one month after transplantation. Hereafter these differences disappeared (Table 3). The serum concentration of parathyroid hormone decreased in the first three months after transplantation in both the steroid-free and control group. Thereafter the serum concentration of parathyroid hormone stabilized, albeit at slightly elevated levels in both groups. In both groups alkaline phosphatase increased slightly compared with baseline.

Table 2. Treatment with corticosteroids, bisphosphonates, vitamin D, and parathyroidectomy during the first 12 months after transplantation.

	Steroid-free N=135	Control N=126
Median (range) cumulative prednisone dose (mg) [a]		
0-3 months	300 (275-3648) [b]	1430 (942-2265)
3-12 months	0 (0-4585) [b]	98 (0-3572)
Number (%) of patients treated with methylprednisolone pulse therapy [c]		
0-3 months	37 (27)	24 (19)
3-12 months	5 (4)	5 (4)
Number (%) of patients treated with calcium carbonate	80 (59)	70 (56)
Number (%) of patients treated with bisphosphonates	11 (8)	7 (6)
Number (%) of patients treated with vitamin D	7 (5)	3 (2)
Number of patients treated with parathyroidectomy after transplantation	3 (2)	4 (3)

[a] Excluding methylprednisolone pulse therapy given in case of a (presumed) acute rejection.

[b] $P < 0.001$.

[c] As given in case of a (presumed) acute rejection.

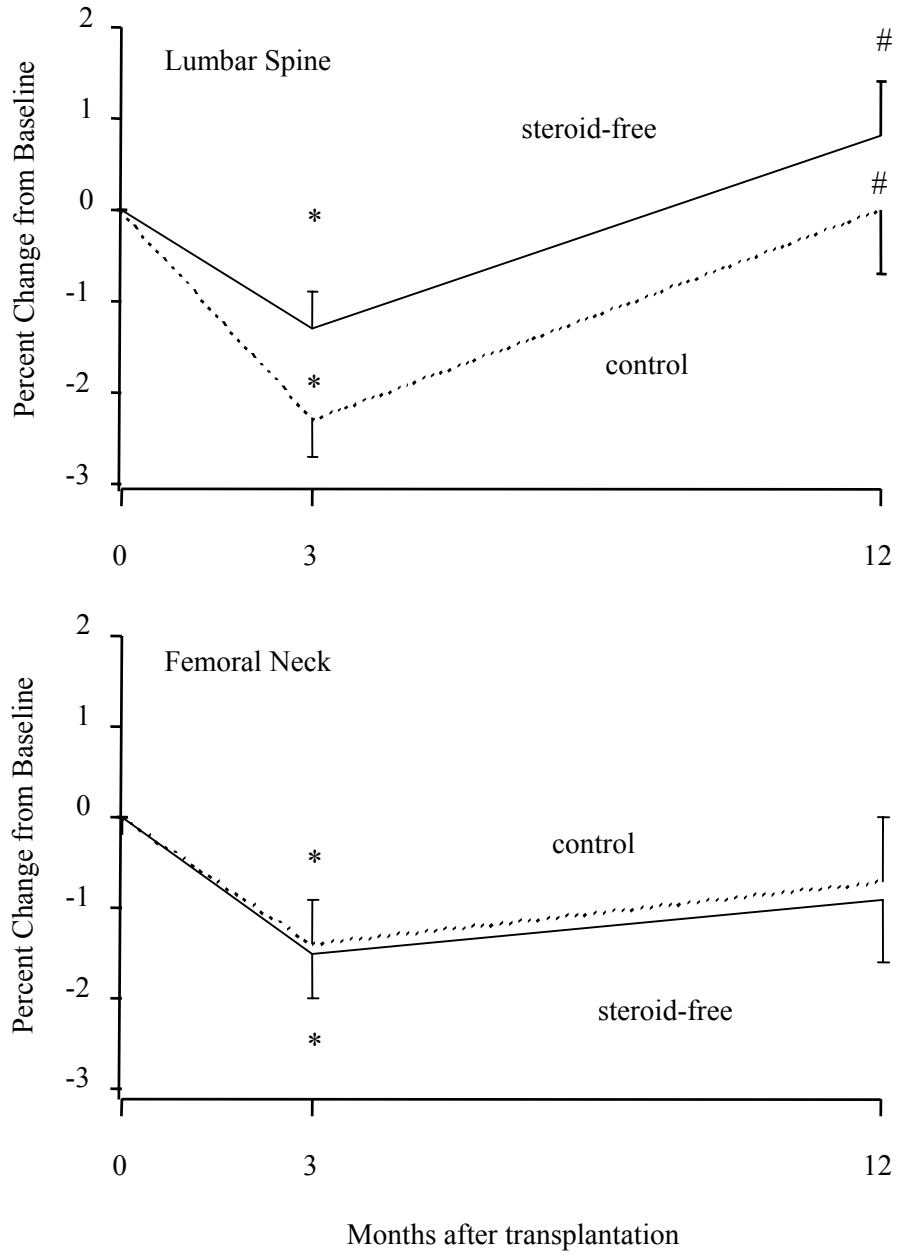

Figure 2. Mean (±SE) change in the bone mineral density of the lumbar spine (top panel), and femoral neck (bottom panel) in the steroid-free and control groups. (*: P< 0.05 versus baseline; # P<0.05 versus month 3).

Table 3. Median (range) serum concentrations of calcium, phosphorus, alkaline phosphatase, and PTH at baseline, months 3 and 12 after transplantation.

	Normal value		Baseline [a]	Month 3	Month 12
Adjusted calcium (mmol/L)	2.25-2.55	Steroid-free	2.52 (1.65-3.53) [c]	2.48 (1.78-3.35) [d]	2.41 (1.93-2.90) [d]
		Control	2.46 (2.06-3.13)	2.49 (2.07-3.19) [d]	2.43 (1.95-3.03) [d]
Phosphorus (mmol/L)	0.8-1.30	Steroid-free	0.78 (0.37-2.51) [c]	0.79 (0.45-2.39) [d]	0.89 (0.43-1.99) [d]
		Control	0.67 (0.30-1.40)	0.81 (0.42-1.30) [d]	0.93 (0.58-1.51) [d]
Alkaline phosphatase (iU/L)	< 120	Steroid-free	105 (34-594)	121 (44-559) [d]	113 (42-322)
		Control	90 (23-956)	102 (49-688) [d]	118 (48-553) [d]
Parathyroid hormone (pmol/L) [b]	1-6.5	Steroid-free	15.6 (0-158)	8.7 (0-112) [d]	9.1 (0-140) [d]
		Control	12.6 (1-160)	8.3 (0-67) [d]	9.1 (0-99) [d]

[a] All values were obtained at one month after transplantation except for parathyroid hormone, which was obtained at the day of transplantation.

[b] For conversion to ng/mL multiply value by 9.5.

[c] $P < 0.05$ between groups.

[d] $P < 0.05$ for comparison with baseline.

Discussion

To our knowledge, this is the first large prospective randomized study that compared the influence of a steroid-free immunosuppressive regimen to a regimen with four months corticosteroid therapy on the change in bone mass during the first year after renal transplantation. The data show that the average change in bone mass during twelve months after transplantation was comparable with both regimens. Apparently the usage of a moderate dose of steroids during four months after transplantation had no important influence on the change in bone mass during the first year after transplantation. The study was population based and did not select patients on the presence of osteoporosis or particular causes of renal osteodystrophy. The scheduled BMD measurements could be performed in the majority of the patients eligible for intention-to-treat analysis. Patients that were not included in the analysis because of lacking BMD measurements were not different with respect to baseline characteristics.

By design of the study, differences between the groups in the change in bone mass should be expected at three months after transplantation. However, despite a clear difference between the groups in the cumulative steroid dose, bone mass decreased at a comparable low rate in both groups at three months after transplantation. A tendency to decreased bone loss of the lumbar spine was found in the steroid-free group. Therefore, a small beneficial effect of the steroid-free immunosuppressive regimen on bone mass is not excluded by our study. Larger study populations are needed to detect such a small difference. Furthermore, we cannot exclude that the change in bone mass has temporarily diverged between the groups later on, due to additional bone loss in the control group during the first period after cessation of steroids. However, no such decrease was seen in a small study at three months after withdrawal of corticosteroids (23). Moreover, the 70 patients in the steroid-free group that did not receive any prednisone maintenance therapy or methylprednisolone pulses had a comparable small but transient decrease in lumbar BMD. We therefore conclude that maintenance therapy with prednisone does not explain the initial transient decrease in BMD, and other factors must be implicated. Besides (partial) resolution of preexistent renal osteodystrophy, immobilization after the operation might be involved (24). The effect on bone mineral density of three days of 100 mg prednisolone intravenously, as administered to the whole study population, is unclear. However, in a cohort of 17 patients with multiple sclerosis, no decrease in BMD was found at two and four months after treatment of 1000 mg methylprednisolone intravenously during three days (25).

In the period between three and twelve months after transplantation bone mineral density recovered, and the average bone mass was comparable to the baseline value in both groups at one year after transplantation. At that time, the immunosuppressive treatment was similar in both groups, and more than 80% of the patients were off steroids. Preservation of bone mass was also reported in previous small or uncontrolled trials in patients treated with a steroid-free regimen (16). In our opinion a steroid-free regimen or a regimen with limited steroid exposure seems the best available option for prevention of accelerated bone loss after transplantation. Considering the tendency to preserved lumbar BMD in the group treated with the steroid-free regimen, this regimen might be preferred in patients at high risk for bone fractures. If treatment with corticosteroids is necessary for more than four months after transplantation, prophylactic treatment should be considered. Treatment with vitamin D and calcium (7) as well as treatment with bisphosphonates (26;27) have been shown to (partially) prevent accelerated bone loss in renal transplant recipients who continued prednisone in combination with cyclosporine and MMF.

Our study has limitations, and the results should be interpreted in the context of its design. BMD is a surrogate marker, and we do not know the effect of the preservation of BMD on the actual bone fracture rate in this population. In our study only three patients experienced a fracture (of the ribs in all cases) during the follow-up period of 12 months, so no conclusions can be drawn on this outcome parameter. However, several studies have shown a similar relationship between low BMD and the risk of fractures in renal transplant recipients as in the normal population (3;4).

In summary, our study indicates that a steroid-free tacrolimus based regimen with induction therapy with daclizumab does not result in a stronger preservation of bone mass than a regimen with four months of steroid therapy. On average, both regimens prevented accelerated bone loss in the first year after renal transplantation.

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Chapter 8

The influence of corticosteroid therapy on quantitative ultrasound parameters of the calcaneus in the first year after renal transplantation

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Abstract

Steroid-related bone loss is a recognized complication after renal transplantation. Quantitative ultrasound (QUS) of bone measures bone structure besides bone mass. We investigated the influence of corticosteroid therapy on QUS parameters during the first year after renal transplantation. A total of 119 renal transplant recipients were randomized to receive either a steroid-free regimen or a regimen with prednisone during four months. In 96 patients (steroid-free: n=49, controls: n=47), QUS parameters of the right calcaneus, and dual energy X-ray absorptiometry (DXA) parameters of the lumbar spine and the right hip were measured at baseline, three, and twelve months after transplantation. Despite a significant difference in steroid use between the two groups, there was no difference between the two groups in QUS and DXA parameters at any time point. BMD of the lumbar spine decreased at three months and recovered hereafter to the baseline value. BMD of the femoral trochanter decreased at three months and remained stable hereafter. In contrast, the QUS parameter broadband ultrasound attenuation (BUA) continued to decrease between three and twelve months after transplantation (steroid-free versus steroids: -4.1% ($P<0.05$) versus -2.6% ($P<0.05$) at twelve months compared with baseline). It is concluded, that the usage of a moderate dose of steroids during four months after renal transplantation did not influence QUS parameters of the calcaneus. While bone mass remained stable or improved between three and twelve months after transplantation, the decrease in BUA seems to reflect a continuing change in bone structure in this population with preexistent renal osteodystrophy.

Introduction

Recipients of a renal transplant are at increased risk to develop bone fractures (1). Renal osteodystrophy and corticosteroid-induced bone loss are important factors involved. Bone mineral density as measured with dual energy X-ray absorptiometry (DXA) has been shown to decrease by 2-7 percent in cyclosporine and prednisone treated patients in the first year after renal transplantation (2-7). However, a low bone mineral density only partly explains the increased fracture rate in renal transplant recipients (8;9). Other factors which are not assessed by DXA, as the microarchitecture and the elasticity of bone are also related with the strength of the bones. Renal osteodystrophy is characterized by a disturbed microarchitecture of bone and is a common finding in histomorphometric studies of patients with renal insufficiency (10;11). Quantitative ultrasound (QUS) measurement of bone is a relatively new non-invasive technique that appears to assess microarchitecture and elasticity in addition to bone mineral density (12). Compared with DXA, QUS is cheaper, is relatively easy to use, takes up less space, and it does not employ ionizing radiation. Most of the commercially available ultrasound devices have been developed for measurement of the calcaneus, as this bone contains a high percentage of trabecular bone mimicking that of the spine and the hip (13). The QUS parameters broadband ultrasound attenuation (BUA) and velocity or speed of sound (SOS) are the two most commonly used. In older healthy women, a low BUA of the calcaneus has been shown to predict hip fractures, independent of DXA (14;15). To our knowledge there are no controlled studies investigating the influence of corticosteroids on QUS parameters in recipients of a renal transplant. It is unclear whether QUS is able to measure other properties of bone compared with DXA in recipients of a renal transplant.

In a prospective randomized study, we compared the influence of a steroid-free immunosuppressive regimen with a regimen with four months corticosteroid therapy on QUS and DXA parameters during the first year after renal transplantation.

Materials and method

Study population and study design

Patients participated in a prospective randomized controlled multicenter trial (16). All 119 patients included at the University Medical Center Nijmegen, the Netherlands were studied. Patients were assigned either to treatment with daclizumab (steroid-free group) or to maintenance treatment with prednisone during the first four months after transplantation (controls). Adult recipients of a first or second transplantation from a living related or cadaveric donor were eligible for this study. In another paper, we have described the exclusion criteria, the details of randomization, and the immunosuppressive protocol (16). In summary, all patients received tacrolimus and mycophenolate mofetil (MMF) as maintenance therapy, and prednisolone 100 mg/day intravenously during the first three days after transplantation. The steroid-free group received the humanized monoclonal antibody daclizumab (1 mg/kg body weight) intravenously one hour before transplantation and once again on day 10-14 after transplantation. The control group received prednisone 0.3 mg/kg/day orally for the first two weeks and thereafter the prednisone dose was gradually tapered to zero in 16 weeks. Acute rejection episodes were primarily treated with methylprednisolone 500-1000 mg intravenously for three consecutive days. In case of interruption of tacrolimus, or a reduction of the MMF dose below 1000 mg/day, or treatment with antilymphocyte therapy, administration of prednisone (dose 0.10-0.15 mg/kg/day) was allowed. Treatment with elementary calcium (500 mg/day) was given, except to patients having hypercalcemia.

Analytical methods

Both QUS and DXA measurements were performed at baseline, at 3, and at 12 months after renal transplantation, preferably at the same visit. QUS measurements of the right heel were performed using the ultrasound bone imaging scanner UBIS 3000 (DMS, Montpellier, France) by scanning the calcaneus in two directions using a pair of 0.5 MHz focussed broadband transducers with a diameter of 25 mm, immersed in a water bath at 30 °C. The acoustic properties broadband ultrasound attenuation (BUA, dB/MHz) and speed of sound (SOS, m/s) were assessed. These ultrasound parameters were measured in a circular region (14 mm) with the lowest attenuation in the posterior part of the calcaneus using a computer algorithm for automatic detection of this region (17). The same operator performed all measurements in order to minimize operator and technical intervariability. The heel of each patient was measured twice with complete repositioning between measurements. If the first

two measurements differed by 10 dB/MHz or more, a third measurement was obtained. Therefore, the definitive result was the mean of two or three measurements. The short-term precision error was calculated at 3.4% for BUA and at 0.38% for SOS (18). Internal acoustic phantoms were scanned before every measurement and the results showed no drift over the period of the study.

BMD was measured by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-4500 (standard array mode). Each subject was measured at the anteroposterior lumbar spine (L1-L4), total hip, femoral neck and trochanter. Standard procedures supplied by the manufacturer for scanning and analyses were followed. Calibration procedures were performed every day and after series of eight scans using the appropriate phantoms provided by the manufacturer. The coefficient of variation for DXA measurements (lumbar phantom scans) was 0.4%. BMD at the lumbar spine, femoral neck, trochanter, and total hip DXA was expressed in g/cm^2 and in Z-scores according to the database delivered by Hologic.

Data and statistical analysis

Results are given as means ± SD unless stated otherwise. QUS and BMD changes in the study period were expressed as a percentage of the baseline value. The statistical analyses were performed on an intention-to-treat basis, including only patients in whom the changes from baseline in QUS parameters could be assessed. Comparison of continuous variables between the groups was performed using unpaired *t* tests. Changes from baseline were assessed with paired t-tests. Log transformation to normalize the distribution of the data, was performed prior to the analysis where appropriate. Chi-square analysis was performed to compare proportions between the two groups. Linear associations between the different parameters were determined by calculation of the Pearson correlation coefficients. For all tests a *P*-value less than 0.05 (two-sided) was considered significant.

Results

Between November 1999 and March 2002, 119 patients were enrolled (Figure 1). Six patients did not receive the allocated regimen for several reasons, leaving 113 patients for intention-to-treat analysis. Additionally, 17 patients were excluded because of failure to complete a baseline and at least one follow-up QUS measurement at the scheduled time. These patients were also excluded from the analysis, leaving 49 patients in the steroid-free group and 47

patients in the control group. The QUS measurements were performed at similar time points in the steroid-free and control group (baseline: 14±4 versus 15±4 days after transplantation; t=3 months: 98±10 versus 99±13 days after transplantation; t=12 months: 370±13 versus 366±15 days after transplantation). The proportion of male patients was higher in the steroid-free group (Table 1). The two groups were not different with respect to the other baseline characteristics as shown in table 1.

Table 1. Baseline characteristics of patients.

Characteristic	Steroid-free (N=49)	Control (N=47)
Age (years)	49±14	47±14
Number of male patients (%)	39 (80) [a]	24 (51)
Number of pre-/ postmenopausal women	3/ 7	10/ 13
Body weight (kg)	75±11	72±13
Body mass index (kg/m^2)	24±3	25±4
Hemodialysis/ peritoneal dialysis/ no dialysis	19/ 22/ 8	27/ 15/ 5
Median (range) duration of dialysis (months) [b]	36 (4-98)	34 (2-83)
Number of patients (%) with previous bone fractures	2 (4)	2 (4)
Median (range) serum parathyroid hormone (pmol/L) [c]	18 (2-158)	14 (2-160)
Bone mineral density of lumbar spine (g/cm^2)	0.973±0.153	0.984±0.157
Z-score	-0.5±1.3	-0.2±1.6
Bone mineral density of total femur (g/cm^2)	0.912±0.147	0.900±0.164
Z-score	-0.5±1.0	-0.4±1.4
Bone mineral density of femoral neck (g/cm^2)	0.806±0.134	0.812±0.136
Z-score	-0.3±1.1	-0.2±1.4
Bone mineral density of femoral trochanter (g/cm^2)	0.714±0.130	0.698±0.129
Z-score	-0.2±1.1	-0.1±1.3
BUA (dB/mHz)	71±16	72±18
SOS (m/s)	1509±36	1504±33

[a] $P<0.05$ versus control group.

[b] Excluding patients transplanted before renal replacement therapy was started.

[c] The normal reference range for serum parathyroid hormone is 1 to 6.5 pmol/L.

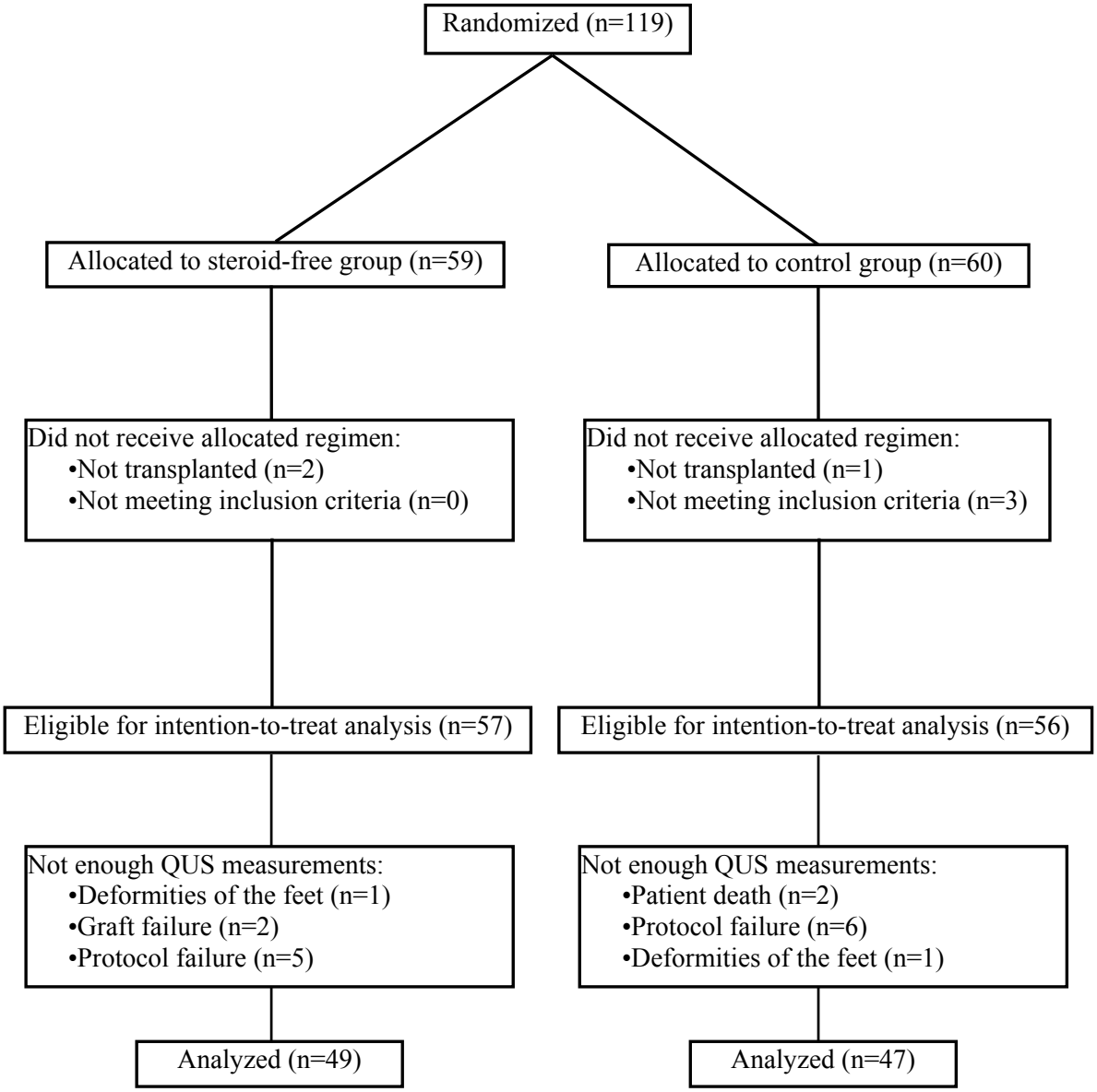

Figure 1. Flow diagram of the patients randomized to one of the treatment groups.

Immunosuppression and acute rejection episodes

In accordance with the study design, the control group received more prednisone, with the largest difference in the first three months after transplantation (Table 2). The number of patients treated with methylprednisolone pulse therapy because of a (presumed) rejection was comparable in both groups (Table 2). In 21 patients (43%) in the steroid-free group, prednisone was started for several clinical reasons (intolerance for MMF (n= 11), intolerance for tacrolimus (n=6), the occurrence of a (severe) acute rejection (n=3), and other causes

(n=1)). At twelve months after transplantation, eight patients in both groups were still using prednisone. There was no difference between the groups in the proportion of patients treated with bisphosphonates, or a parathyroidectomy in the first year after transplantation (Table 2).

Table 2. Treatment with corticosteroids, bisphosphonates, and parathyroidectomy during the first 12 months after transplantation.

	Steroid-free (N=49)	Control (N=47)
Median (range) cumulative prednisone dose (mg) [a]		
0-3 months	300 (300-2385) [b]	1412 (942-2280)
3-12 months	0 (0-5165) [b]	95 (26-3012)
Number (%) of patients treated with methylprednisolone pulse therapy [c]		
0-3 months	15 (31)	12 (26)
3-12 months	1 (2)	1 (2)
Number (%) of patients treated with bisphosphonates [d]	10 (20)	4 (9)
Number of patients treated with parathyroidectomy after transplantation	3 (6)	3 (6)

[a] Excluding methylprednisolone pulse therapy given in case of a (presumed) acute rejection.

[b] $P<0.001$

[c] As given in case of a (presumed) acute rejection.

[d] As given in case of hypercalcemia

BMD and QUS changes

The mean (±SE) changes in BMD and QUS parameters during the first year after transplantation are shown in figure 2, 3 and 4. There were no differences between the groups in the changes in BMD or QUS parameters at any time point (Table 3).

However, compared with baseline, significant changes in BMD and QUS parameters were detected in both groups. In the control group, BMD of the lumbar spine and the total hip

decreased in the first three months after transplantation (-2.2±3.1%; $P<0.001$ and -1.2±3.3%; $P<0.05$, respectively), and returned to the baseline value at twelve months after transplantation (Figure 2). In the steroid-free group, no significant changes were found for these parameters at three months (lumbar spine: -1.1±4.3%; P=0.09 and total hip: -0.5±4.1%; NS) and at twelve months after transplantation (Figure 2). The BMD of the femoral trochanter decreased in the steroid-free and control group at three months after transplantation (-1.4±5.3%; $P<0.05$ and -2.0±3.9%; $P<0.01$) and remained below the baseline level in both groups at twelve months after transplantation (-2.2±8.6%; $P<0.05$ versus -1.8±7.8%; $P<0.05$) (Figure 3).

The QUS parameter BUA was significantly lower at twelve months after transplantation compared with baseline in the steroid-free and control group (-4.1±10.3% ($P<0.01$) and -2.6±10.0% ($P<0.05$), respectively (Figure 4). In contrast to the DXA parameters, BUA continued to decrease ($P<0.05$) between three and twelve months after transplantation in both groups. SOS decreased significantly in the steroid-free group at three months after transplantation (-0.3±0.8%; $P<0.01$), and returned to the baseline value at twelve months after transplantation. No significant changes in SOS were found in the control group.

Exclusion of the patients in the steroid-free group who were treated with steroids during follow-up did not change the results. In addition, correction for gender with covariance analysis did not change the results.

Table 3. Percentage difference (95% confidence interval of difference) between the groups in the change in BMD and QUS parameters at three and twelve months after transplantation.

	Percentage difference between the steroid-free and control group (95% confidence interval of difference)	
	Three months	Twelve months
Lumbar spine BMD	+1.1 (-0.5 to +2.7)	+0.4 (-2.0 to +2.8)
Total hip BMD	+0.8 (-0.8 to +2.4)	-0.0 (-3.0 to +2.9)
Femoral neck BMD	-0.4 (-2.6 to +1.9)	-0.4 (-4.2 to +3.4)
Femoral trochanter BMD	+0.6 (-1.4 to +2.6)	-0.4 (-3.9 to +3.1)
BUA	-0.7 (-2.9 to +1.6)	-1.5 (-5.7 to +2.7)
SOS	-0.14 (-0.49 to +0.21)	-0.25 (-0.79 to +0.28)

Correlation between (changes in) QUS and DXA parameters

At baseline the correlation between BUA and BMD was moderate ranging from r=0.55 for the femoral neck to r=0.66 for the femoral trochanter (P<0.001). There was no significant correlation between the percent changes in the BUA and the percent changes in BMD between zero and three months after transplantation. Regarding the entire study period from zero to twelve months after transplantation, the percent changes in BUA were significantly correlated with the percent changes in BMD (r= 0.32, r= 0.54, r= 0.52, r=0.58, all P<0.01, for the lumbar spine, total hip, femoral neck, and trochanter BMD, respectively). Between three and twelve months after transplantation, the percent changes in BUA were also significantly correlated with the percent changes in BMD (r= 0.30, r= 0.50, r= 0.40, r=0.51, all P<0.01, for the lumbar spine, total hip, femoral neck, and trochanter BMD, respectively).

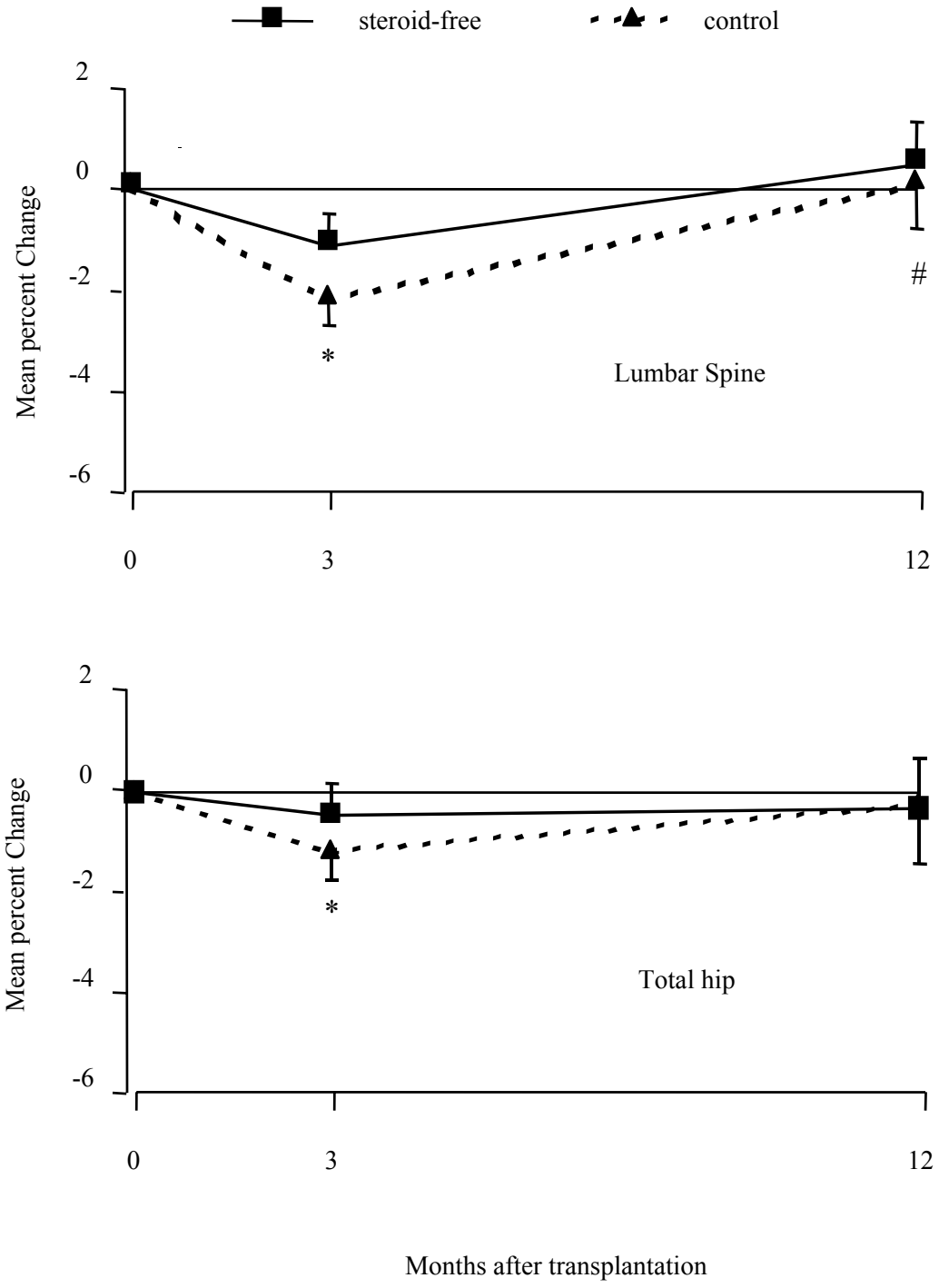

Figure 2. Mean percent change (±SE) from baseline in the bone mineral density of the lumbar spine (top panel), and total hip (bottom panel) in the steroid-free and control groups.
(*: $P< 0.05$ versus baseline; #: $P<0.05$ versus month 3).

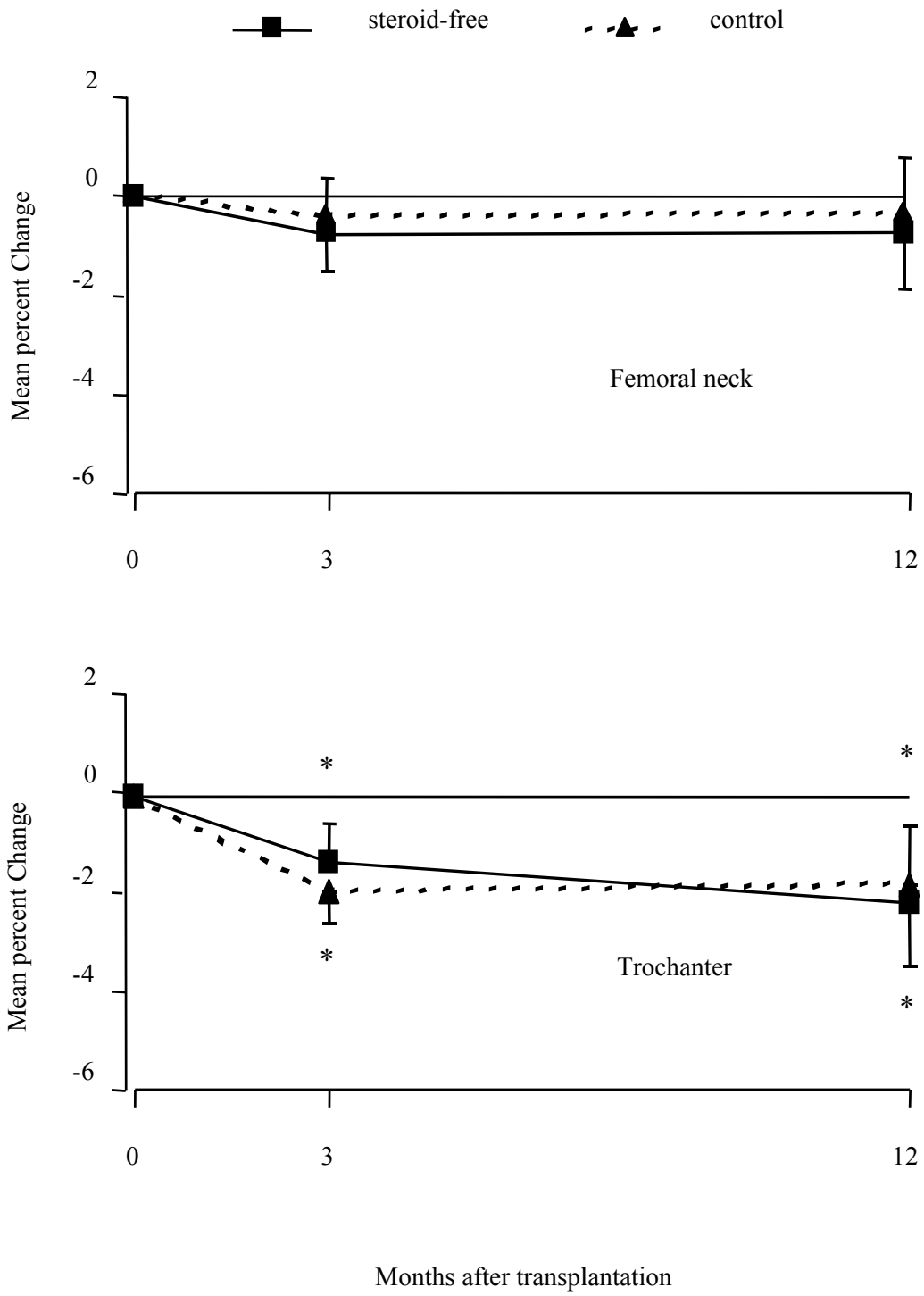

Figure 3. Mean percent change (±SE) from baseline in the bone mineral density of the femoral neck (top panel), and femoral trochanter (bottom panel) in the steroid-free and control groups.
(*: $P< 0.05$ versus baseline).

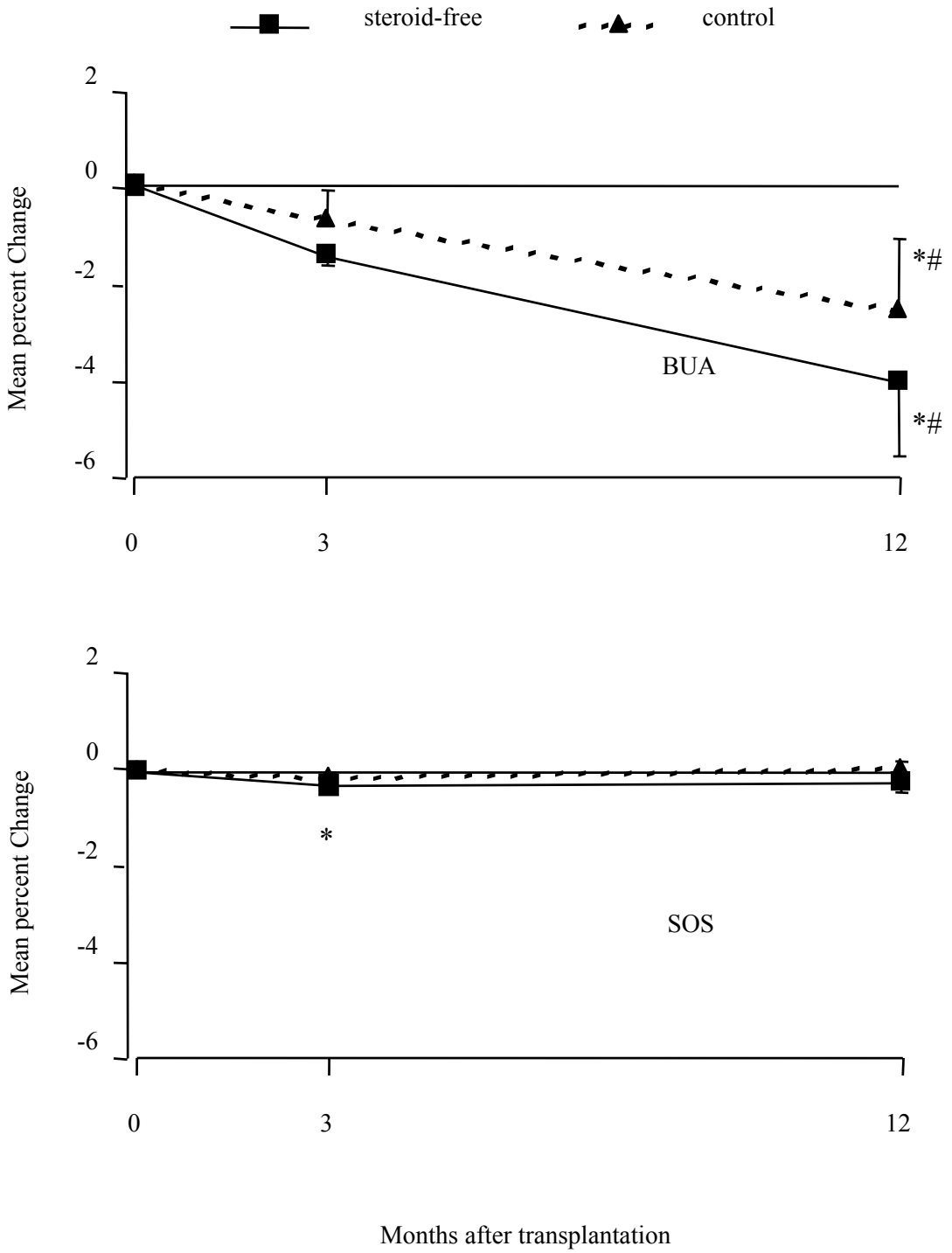

Figure 4. Mean percent change (±SE) from baseline in QUS parameters in the steroid-free and control groups. BUA, broadband ultrasound attenuation (top panel); SOS, speed of sound (bottom panel). (*: $P< 0.05$ versus baseline; #: $P<0.05$ versus month 3).

Discussion

This is the first prospective randomized study comparing the influence of a steroid-free immunosuppressive regimen to a regimen with limited steroid exposure on quantitative ultrasound parameters of the calcaneus after renal transplantation. The data show, that the average change in QUS parameters was comparable with both regimens. Apparently, no additional influence of a moderate dose of steroids was found on QUS parameters during twelve months after transplantation. So far, cross-sectional studies have reported a decrease (19-23) in QUS parameters in other populations using glucocorticoids. As QUS parameters are highly influenced by bone mass (24), the decreased QUS parameters reported in these studies parallel the well-known decrease in bone mass of patients treated with glucocorticoids. In this longitudinal study however, the course of BMD as assessed by DXA and QUS was not influenced by a moderate dose of steroids. Moreover, the microarchitecture and elasticity of bone as measured by QUS, were not influenced by about ±1200 mg of prednisone given during the first year after transplantation.

Of interest, the time course of the changes in the QUS parameter BUA seemed different from the changes in DXA parameters. While DXA parameters remained more or less stable or decreased temporary, the average BUA continued to decrease in both groups between three and twelve months after transplantation. We consider the decrease of BUA during the first year after transplantation as a 'real change', since the measured difference exceeded the precision error, and the calibration procedures showed no drift of the QUS device during the study. Furthermore, the annual decrease of BUA in our study is clearly larger than the reported annual rate of change in pre- and postmenopausal women (25). BUA is related to bone mineral density as well as to the microarchitecture of trabecular bone (12). The decrease in BUA might be the result of a regional decrease in BMD of the calcaneus. We did not measure the BMD of the calcaneus, but considering the stable BMD of the femoral neck and trochanter, or even the improved BMD of the lumbar spine between three and twelve months after transplantation, it is not likely that the continued decrease in BUA can be explained by a decrease in calcaneal BMD. Other longitudinal studies demonstrated that changes in BMD of the calcaneus followed the changes in BMD of the lumbar spine and the femoral neck (26;27). Individually, the changes in BUA were only moderately correlated with the changes in BMD. The variation in the change in BUA was explained by the change in BMD of the lumbar spine and hip for maximal 34 percent. This underlines that the changes in BUA are only marginally explained by changes in bone mineral density in this population. Since BUA is related to

density as well as to bone structure (12), it is possible that the average change in BUA found in this study reflect a change in bone structure but not density after renal transplantation. A cross-sectional study in renal transplant recipients, demonstrated that BUA and SOS correlated to trabecular bone histomorphometry (9), however, it is unclear from this study if this relation was independent of BMD. Prospective studies with respect to the changes in QUS parameters, BMD, and bone structure as assessed by bone histomorphometry may add additional information regarding the change of bone properties after transplantation.

The relative change in SOS in our study was very marginal, which is inherent to the parameter. The change in SOS during aging from 20 to 80 years is limited to ±5 percent of the baseline value (25). The pattern of the changes in the QUS parameter SOS was comparable to the changes in BMD of the lumbar spine and total hip, although changes were only detected in the steroid-free group.

So far, only one prospective, uncontrolled study addressed the changes in QUS parameters after renal transplantation (28). In contrast to our study, no changes in the QUS parameters BUA and SOS of the calcaneus were found in 46 cyclosporine, mycophenolate mofetil and prednisone treated patients during the first six months after transplantation. In this study, a QUS device without imaging techniques was used, while in our study QUS parameters were measured with an imaging device in the region of lowest attenuation. Furthermore, the follow-up of this study was shorter, and the sample size smaller compared with our study. These aspects influence the ability to measure a longitudinal change in QUS parameters. It is unclear whether BUA in our study population will continue to decrease at longer follow-up. In two cross-sectional studies (29;30) there was no relation reported between the time after transplantation and QUS parameters, which might indicate that BUA stabilizes at longer follow-up. Besides the fact that these are cross-sectional data, both studies were also performed with a non-imaging device.
A low BUA of the calcaneus has been shown to predict hip fractures (14;15;31). Information with regard to the fracture incidence is limited in renal transplant recipients. In one cross-sectional study, a low SOS was associated with increased fracture incidence in female renal transplant recipients (9). However, no difference was found in BUA between patients with or without a fracture. Our study delivers no additional information, since no fractures occurred during the first year after renal transplantation.

In conclusion, QUS parameters of the calcaneus are not influenced by a limited dose of corticosteroids during the first four months after renal transplantation. In contrast to DXA parameters, the average BUA continued to decrease after transplantation in both groups. This continued decrease in BUA might be a reflection of a change in bone structure in this population with preexistent renal osteodystrophy. At present, the relevance of this finding is unclear.

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Chapter 9

Summary

In the last two decades, the results of renal transplantation have tremendously improved by the availability of new and potent immunosuppressive drugs. However, recipients of a renal transplant often experience several side effects specifically related to these immunosuppressive drugs, such as an increase in cardiovascular risk factors leading to cardiovascular morbidity, increased susceptibility to infections and malignancies, and increased bone loss leading to bone fractures. Therefore, finding the balance between adequate immunosuppression and the occurrence of side effects is an important clinical problem.

Monoclonal antibodies directed against the alpha chain of the interleukin-2 receptor (IL-2Rα), are drugs with a highly specific immunosuppressive mechanism of action. Induction therapy with these agents was not associated with an increased incidence of adverse events, and seems an important advance in the above-mentioned balance. Therefore these agents may prove particularly advantageous in the treatment of transplant recipients.
In *chapter two*, we provide an overview of the available monoclonal antibodies against IL-2Rα and its use in renal transplantation. Basiliximab is a chimeric monoclonal antibody, daclizumab a humanized antibody directed against IL-2Rα. In our studies we have used daclizumab as induction therapy. Daclizumab has been traditionally administered in a five-dose regimen after transplantation. The first infusion is administered intravenously one hour before transplantation and once again every two weeks. So patients have to come to the hospital to receive these infusions. Besides, daclizumab is very expensive. On theoretical grounds, we have decided to administer only two doses of daclizumab after transplantation. In *chapter three* we demonstrated that a two-dose regimen of daclizumab in combination with tacrolimus and mycophenolate mofetil resulted in a blockade of the IL-2Rα-chain, which lasted for more than 10 weeks after transplantation. The two-dose regimen of daclizumab in combination with tacrolimus and mycophenolate mofetil seemed sufficient for adequate immunosuppression as was suggested by a low incidence of acute rejection (13%).

In the following two chapters, the development of an alternative method for monitoring IL-2Rα blockade of lymphocytes is described. Soluble IL-2Rα is the truncated form of the alpha chain of the IL-2 receptor and is derived from proteolytic cleavage of membrane bound IL-2Rα. Soluble IL-2Rα is filtered by the kidney and excreted with the urine. We hypothesized that excess daclizumab would bind to the IL-2Rα on peripheral lymphocytes, as well as to soluble IL-2Rα in serum. In the following experiments we observed a decreased renal

excretion of IL-2Rα during daclizumab treatment (c*hapter four).* It seems likely that binding of daclizumab to soluble IL-2Rα leads to the formation of a large complex thus preventing glomerular leakage of soluble IL-2Rα. Thus we concluded that measurement of urinary soluble IL-2Rα may provide information on the concentration of anti-IL-2Rα mAb in serum. We have compared urinary measurements of soluble IL-2Rα with the golden standard, flow cytometry (*chapter five);* from the data we can conclude that IL-2Rα blockade of peripheral lymphocytes after treatment with daclizumab can reliably be assessed by calculation of the fractional excretion of soluble IL-2Rα. This method is easier to use, compared with the current gold standard, flow cytometric analysis of IL-2Rα on lymphocytes.

In *chapter six and seven,* we describe our studies specifically aimed at the evaluation of the use of daclizumab as replacement therapy for steroids after renal transplantation. The use of corticosteroids is associated with several well-documented side effects such as hypertension, hyperlipidemia, new-onset diabetes mellitus, infections, and accelerated bone loss. In a prospective, randomized, multicenter study the avoidance of steroids by the administration of two doses of daclizumab was compared with four months of corticosteroid treatment in 364 renal transplant recipients on a tacrolimus and mycophenolate mofetil-based immunosuppressive regimen. We could demonstrate that treatment with two doses of daclizumab enabled the use of a steroid-free immunosuppressive regimen after renal transplantation (*chapter six*). There was no difference in the incidence of biopsy-proven acute rejection. The steroid-free immunosuppressive regimen had a temporary beneficial effect on several cardiovascular risk factors (lower blood pressure, lower serum cholesterol, and less new-onset diabetes mellitus). This beneficial effect should be balanced against the increased costs associated with the steroid-free regimen.
In *chapter seven*, we describe that the steroid-free regimen as well as the regimen with limited steroid exposure prevented accelerated bone loss in the first year after transplantation. Four months of corticosteroid therapy had no important influence on the change in bone mass during the first year after renal transplantation.

A low bone mineral density explains the increased fracture incidence in renal transplant recipients to a minor extent. The microarchitecture and the elasticity of bone are important factors involved in the strength of the bones. Dual energy X-ray absorptiometry, the standard method for the assessment of bone mineral density does not assess the structure of bone.

Quantitative ultrasound of bone has been shown to measure bone structure besides bone mineral density. In a sub-population of the earlier mentioned prospective randomized study we have compared these methods. We noted that four months of corticosteroid therapy did not influence quantitative ultrasound parameters of the calcaneus (*chapter eight*). However, quantitative ultrasound of bone seemed to measure different properties of bone compared with dual energy X-ray absorptiometry. While bone mineral density remained stable or improved between three and twelve months after transplantation, the quantitative ultrasound parameter broadband ultrasound attenuation continued to decrease in this period. The changes in the quantitative ultrasound parameter broadband ultrasound attenuation seem to reflect a continuing change in bone structure in this population with preexistent renal osteodystrophy.

Chapter 10

Samenvatting

Na transplantatie van een orgaan van een niet-identieke donor, zal het afweersysteem van de ontvanger van dit orgaan een reactie in gang zetten om dit orgaan af te stoten. Om afstoting te voorkomen moet deze natuurlijke afweerreactie met medicijnen worden onderdrukt. Ongeveer 20 jaar geleden was acute afstoting een belangrijke oorzaak van vroegtijdig verlies van het niertransplantaat. Destijds werden de patiënten behandeld met de weerstandverlagende medicijnen azathioprine en prednison. Eén jaar na de transplantatie functioneerde de nier maar bij 60-65% van de patiënten. Met de toepassing van nieuwe en krachtige medicijnen als ciclosporine, tacrolimus en mycofenolaat mofetil zijn de resultaten van niertransplantatie sterk verbeterd. Met een combinatie van deze medicijnen (tot nu toe altijd met prednison) heeft minder dan 25% van de patiënten een acute afstotingsreactie, en één jaar na de transplantatie functioneert de nier bij 80 tot 100% van de patiënten. Echter, veel patiënten met een niertransplantaat hebben bijwerkingen van deze medicijnen. Door langdurig gebruik van de weerstandverlagende medicijnen hebben deze patiënten een grotere kans op het krijgen van hart- en vaatziekten, ontstekingen, kwaadaardige ziekten en botbreuken. In ons streven om het afweersysteem optimaal te onderdrukken moeten we de voordelen van deze medicijnen (minder afstotingsreacties) afwegen tegenover de bijwerkingen. Recent zijn weerstandverlagende medicijnen beschikbaar gekomen, die op zeer specifiek wijze de afweer onderdrukken zonder duidelijke bijwerkingen. Voorbeelden hiervan zijn de monoklonale antistoffen basiliximab en daclizumab, welke de interleukine-2 receptor op de afweercellen blokkeren. Deze antistoffen worden altijd in combinatie met andere weerstandverlagende medicijnen gebruikt. In dit proefschrift beschrijven we onze ervaringen met daclizumab. Met name waren wij geïnteresseerd of het mogelijk is om daclizumab in de plaats van prednison te gebruiken na niertransplantatie.

In *hoofdstuk twee* van dit proefschrift, wordt een overzicht gegeven van het gebruik van de interleukine-2 receptor blokkerende antistoffen na niertransplantatie. Tot nu toe werd daclizumab in vijf doses gegeven met een infuus. Theoretisch gezien lijkt een vermindering van het aantal doses geen invloed te hebben op de effectiviteit van daclizumab. Derhalve hebben wij besloten om de dosering daclizumab te verminderen tot twee doses na niertransplantatie. Om te weten of daclizumab na behandeling nog in het bloed van de patiënt aanwezig is, kan worden gemeten of de interleukine-2 receptor op de afweercellen geblokkeerd is. In *hoofdstuk drie* beschrijven wij, dat na de behandeling met twee infusen daclizumab de interleukine-2 receptor op de afweercellen meer dan 10 weken na de transplantatie geblokkeerd blijft. Daclizumab werd samen met twee andere weerstandverlagende medicijnen (tacrolimus

en mycofenolaat mofetil) gegeven. Met deze combinatie medicijnen had slechts 13% van de patiënten een acute afstoting.

De standaardmethode om vast te stellen of de interleukine-2 receptor op de afweercellen is geblokkeerd, is technisch ingewikkeld en kost veel tijd. Wij hebben een alternatief voor deze standaardmethode ontwikkeld. Hiervoor hebben we de invloed van daclizumab op oplosbaar interleukine-2 receptor in het bloed onderzocht. Oplosbaar interleukine-2 receptor wordt normaal door de nier uit het bloed gefilterd en met de urine uitgescheiden. Het blijkt, dat daclizumab samen met oplosbaar interleukine-2 receptor een complex vormt, dat te groot is om gefilterd te worden door de nier *(hoofdstuk vier)*. Zolang daclizumab in een voldoende hoge concentratie in het bloed aanwezig is, wordt oplosbaar interleukine-2 receptor niet met de urine uitgescheiden. Met het dalen van de concentratie van daclizumab in het bloed, wordt oplosbaar interleukine-2 receptor weer in de urine gevonden. Het meten van oplosbaar interleukine-2 receptor in de urine geeft dus informatie over de aanwezigheid van daclizumab in het bloed. In *hoofdstuk vijf* beschrijven wij, dat het meten van oplosbaar interleukine-2 receptor in de urine een betrouwbare manier is om vast te stellen of de interleukine-2 receptor op de afweercellen geblokkeerd is na behandeling met daclizumab. Deze methode is eenvoudiger dan de standaardmethode om dit vast te stellen, en mogelijk zelfs iets beter.

In *hoofdstuk zes* beschrijven we ons onderzoek waaruit blijkt dat het mogelijk is om daclizumab in plaats van prednison na niertransplantatie te geven. Dit onderzoek vond plaats bij 364 patiënten die een niertransplantatie ondergingen in de academische ziekenhuizen van Nijmegen, Rotterdam, Utrecht of Maastricht. Er werd voor de transplantatie geloot, waarbij de ene helft van de patiënten twee infusen met daclizumab kreeg, en de andere helft gedurende vier maanden prednison. Alle patiënten kregen eveneens de weerstandverlagende medicijnen tacrolimus en mycophenolate mofetil. Patiënten die behandeld werden met daclizumab hadden even vaak een afstotingsreactie als patiënten die behandeld werden met prednison. Patiënten die behandeld werden met daclizumab hadden tijdelijk een lagere bloeddruk, een lager cholesterol en een kleinere kans om suikerziekte te krijgen. De behandeling met daclizumab was echter duidelijk duurder dan de behandeling met prednison. In *hoofdstuk zeven*, beschrijven we dat de botdichtheid, gemiddeld genomen, in beide groepen niet veranderde. Behandeling met een matige dosis prednison gedurende vier maanden gaf geen duidelijke toename van botontkalking in het eerste jaar na niertransplantatie.

Bekend is dat patienten met een lage botdichtheid (osteoporose) eerder hun botten breken. Niet alle patiënten met een lage botdichtheid echter breken hun botten, en er zijn patiënten met een hoge botdichtheid die juist wel hun botten breken. Behalve de botdichtheid, zijn andere factoren zoals de structuur en de elasticiteit van het bot van belang voor de sterkte van het bot. Bekend is bij patiënten met een gestoorde nierfunctie, dat de structuur van het bot verandert. Met de standaardmethode voor het bepalen van de botdichtheid wordt geen rekening gehouden met veranderingen in de structuur van het bot. Kwantitative echo van het bot lijkt zowel de veranderingen in de structuur als de botdichtheid te meten. Bij 119 patiënten hebben we gelijktijdig met de standaardmethode om de botdichtheid vast te stellen een botecho gedaan. In *hoofdstuk acht* beschrijven we dat behandeling gedurende vier maanden met prednison ook geen duidelijke invloed had op de uitkomst van de botecho metingen van het hielbeen. Echter, de botecho lijkt wel iets anders te meten dan de standaardmethode voor het bepalen van de botdichtheid. Terwijl de botdichtheid stabiel bleef of zelfs verbeterde tussen drie en twaalf maanden na transplantatie, nam de uitkomst van de botecho metingen alleen maar verder af in deze periode. Dit verschil met de standaardmethode is een aanwijzing, dat de botecho veranderingen in de botstructuur na niertransplantatie meet.

Conclusie

Met de huidige weerstandverlagende medicijnen heeft het merendeel van de patiënten één jaar na niertransplantatie een goede nierfunctie. Bijwerkingen van de weerstandverlagende medicijnen zijn voor veel patiënten een belangrijk probleem. De onderzoeken beschreven in dit proefschrift geven aan dat een beperkte dosis met de interleukine-2 receptor blokkerende antistof daclizumab, het mogelijk maakt om veilig zonder prednison een niertransplantatie te ondergaan. In vergelijking met de behandeling met een lage dosering prednison gedurende vier maanden is het voordeel van deze prednisonloze behandeling echter beperkt. De prednisonloze behandeling leidt tot een tijdelijke vermindering in een aantal risicofactoren voor hart- en vaatziekten, maar is tevens veel duurder dan het geven van prednison gedurende vier maanden.

Chapter 11

General discussion

In the last decades the results of renal transplantation have improved (1), at least partially as the result of the use of new and powerful immunosuppressive agents. Monoclonal antibodies against the alpha chain of the interleukin-2 receptor (IL-2Rα) are a new class of immunosuppressive agents with a highly specific mode of action. Two preparations are available for use, basiliximab and daclizumab, a chimeric and a humanized antibody, respectively. The first clinical studies have demonstrated the efficacy of these agents in reducing the rate of acute rejection. Most remarkably, the use of these agents was not associated with an increase in adverse events. Therefore it was hoped that these agents might prove an advantage in the management of renal allograft recipients. In our studies we have used daclizumab. In the present chapter we discuss the results of our studies in the context of our current knowledge on immunosuppressive treatment after renal transplantation. In general, studies have analyzed the benefits of anti-IL-2Rα therapy either added to standard immunosuppressive regimens (additional immunosuppression) or as replacement of one or more components of the standard immunosuppressive therapy (minimization of side effects). With respect to the side effects, we specifically discuss our experience with bone loss after transplantation.

Use of anti-IL-2Rα therapy as additional immunosuppression

Acute rejection

Anti-IL-2Rα therapy has been demonstrated to decrease the acute rejection rate by ± 40 to 60% when added to cyclosporine combined with prednisone (2-4), and azathioprine (5;6), or tacrolimus, mycophenolate mofetil (MMF), and prednisone (7). In an underpowered trial, a tendency towards a decreased incidence of acute rejection was found after addition of basiliximab to cyclosporine, MMF and prednisone (8). There are no controlled studies that have evaluated the benefits of anti-IL-2Rα therapy when added to sirolimus-based immunosuppressive regimens.

Long-term graft survival

In the individual controlled trials, the one-year graft survival was not improved by induction therapy with anti-IL-2Rα antibodies (9). A study that combined the long-term follow-up data of two pivotal trials with daclizumab concluded that the graft survival at three year after

transplantation was also not better in the daclizumab-treated groups (10). Thus, at present there is no evidence that the forty percent decrease in the acute rejection rate after induction therapy with daclizumab translates in an improvement of long-term graft survival. Although there are no data, it is highly unlikely that conclusions will be different for induction therapy with basiliximab. This lack of a beneficial effect on graft survival challenges the view that it is worthwhile to increase the immunosuppressive load with anti-IL-2Rα therapy. Admittedly, longer follow-up might be required to detect a (small) survival advantage with anti-IL-2Rα therapy.

Adverse events

In the first studies anti-IL-2Rα therapy was very well tolerated (7;9). There was no increase in the number of adverse events. The data of the long-term follow-up study were also reassuring, with no increased risk to develop malignancies or infections in the daclizumab group (10). However, anaphylactic reactions have been described after (repeated) treatment with the chimeric antibody basiliximab (11-14). Three possible cases of hypersensitivity reactions have also been reported in patients using daclizumab (15). Close monitoring is required to detect less frequently occurring side effects that may become apparent only when anti-IL-2Rα therapy is used in a larger population.

Limited dose regimen

In the pivotal studies daclizumab was used in a five-dose regimen. In our studies we have administered daclizumab in a two-dose regimen with apparent success (rejection rate ±15%). Others have shown that even one dose of daclizumab decreased the acute rejection rate by ± 60% (7). Therefore it seems feasible to limit the dose of daclizumab, which increases the easiness of administration and probably also the cost-effectiveness of this agent. We must admit that there are no controlled studies directly comparing the efficacy of different dose-regimens in renal transplantation. Such a comparison has only been performed after simultaneous kidney-pancreas transplantation (16). In the latter study, no difference in acute rejection rate was found between a two and five-dose regimen (16).

Use of anti-IL-2Rα therapy to minimize side effects

The lack of toxicity of anti-IL-2Rα therapy has been the rationale to develop immunosuppressive regimens in which anti-IL-2Rα therapy are used to replace other, more toxic immunosuppressive drugs. It is obvious, that the efficacy of such regimens must be determined in randomized controlled clinical studies.

Comparison of anti-IL-2Rα therapy with antilymphocyte therapy

Both polyclonal and monoclonal antilymphocyte antibodies are effective in delaying or preventing the onset of acute rejection. These agents even seem to prolong long-term graft survival in presensitized patients (17). However, the benefits of antilymphocyte therapy remain controversial. Moreover, antilymphocyte therapy is poorly tolerated due to the cytokine-release syndrome (18), and is clearly associated with an increased incidence of opportunistic infections and lymphoproliferative disease (19). A recent study revealed that antilymphocyte therapy is associated with an increased risk of death due to cardiovascular diseases, infections, and malignancies (20). Therefore, prophylactic use of antilymphocyte therapy is commonly restricted to patients at high immunological risk for rejection. Two small controlled studies in renal allograft recipients have compared anti-IL-2Rα therapy with antithymocyte globulin (21;22). In these studies, basiliximab and early initiation of cyclosporine was compared with antithymocyte globulin and initiation of cyclosporine after establishment of renal function. All patients additionally received MMF and prednisone. In both studies, the incidence of acute rejection was comparable in the two groups. One retrospective study has evaluated the effects of daclizumab and OKT3 induction therapy in patients treated with tacrolimus, MMF and prednisone. The acute rejection incidence was significantly lower (2%) in the daclizumab group compared with the OKT3 group (7%) (23). In all studies, adverse events (i.e. infections) were more frequently reported in antilymphocyte therapy treated patients. These data suggest that anti-IL-2Rα therapy may be preferable to antilymphocyte therapy for rejection prevention. Of note, the mentioned studies were not restricted to patients at high immunological risk.

Comparison of anti-IL-2Rα therapy with MMF

MMF has been shown to decrease the acute rejection rate by ± 50% when added to a cyclosporine-based regimen (24). In contrast to anti-IL-2Rα therapy, use of MMF has been shown to maintain long-term allograft function after renal transplantation (25;26). This long-term effect was achieved by continuation of MMF for at least one year after transplantation.

Well-known adverse events of the use of MMF are gastrointestinal intolerance (27), bone marrow depression, and an increased susceptibility to symptomatic CMV infection (28). Replacement of MMF by anti-IL-2Rα therapy would avoid these adverse events, thus improving the quality of life in a proportion of the patients. However, there are no studies comparing anti-IL-2Rα (induction) therapy with (continuous) treatment with MMF.

Comparison of anti-IL-2Rα therapy with calcineurin inhibitors

The use of the calcineurin inhibitors cyclosporine (29) and tacrolimus (30) has significantly improved the results of organ transplantation. However, both calcineurin inhibitors frequently cause side effects, some acutely, others apparent after more chronic use only. Acutely these drugs can cause afferent arteriolar vasoconstriction leading to a reversible dysfunction of the renal allograft. Chronic administration of calcineurin inhibitors may cause interstitial fibrosis leading to an irreversible dysfunction of the renal graft (31;32). Both drugs increase cardiovascular risk factors such as hypertension, hyperlipidemia, and hyperglycemia, an important issue in view of the increased cardiovascular morbidity and mortality in this population (33). Withdrawal of cyclosporine has been shown to improve renal graft function and cardiovascular risk factors (34-37). In a meta-analysis, cyclosporine withdrawal was associated with an increase in acute rejection rates, although short-term graft survival was not impaired (38).

Avoidance of calcineurin inhibitors after transplantation has been the focus of several studies. In phase II studies, induction therapy with daclizumab in combination with MMF and prednisone (39;40) resulted in an acute rejection rate of 38% and 50%. This rejection rate is disproportional high, when compared to studies using a calcineurin inhibitor in combination with MMF and prednisone. It is therefore evident, that anti-IL-2Rα therapy is less effective than a calcineurin inhibitor to prevent acute rejection episodes. Graft survival however was excellent in these studies, and long-term follow-up data have to be awaited.

In a small randomized study, the acute rejection rate was 16% in patients treated with a combination of basiliximab, sirolimus, MMF and prednisone, comparable to the 6% rejection rate in patients treated with a combination of cyclosporine, MMF and prednisone (41). Other (small) studies have compared sirolimus- and cyclosporine-based regimens, all without anti-IL-2Rα therapy, and observed a comparable incidence of acute rejection with both regimens (42;43). These studies suggest that sirolimus is effective for prevention of acute rejection, and thus may replace the calcineurin inhibitors. However, as stated above, it is unclear if anti-IL-

2Rα therapy is of additional value when added to treatment with sirolimus after transplantation.

Avoidance of calcineurin inhibitors is especially of interest in patients with a kidney from a marginal donor. These patients are particularly susceptible to the acute hemodynamic side effects of the calcineurin inhibitors, leading to a delayed function of the allograft. Uncontrolled observations have suggested that anti-IL-2Rα therapy (44) combined with sirolimus (45) may delay or even avoid (46) the use of calcineurin inhibitors. Promising results were also obtained after conversion from a calcineurin inhibitor-based regimen to a sirolimus-based regimen combined with anti-IL-2Rα therapy in patients with hemolytic uremic syndrome after transplantation (47). However, treatment with sirolimus has also been associated with an increase in delayed graft function (48;49), so controlled trials specifically evaluating possible advantages of sirolimus in patients with delayed graft function or a hemolytic uremic syndrome are needed.

Comparison of anti-IL-2Rα therapy with corticosteroids

Corticosteroid therapy has been the mainstay for rejection prophylaxis after transplantation. The use of corticosteroids is associated with several well-documented side effects such as hypertension, hyperlipidemia, new-onset diabetes mellitus, infections, a cushingoid habitus, skin bruising, and accelerated bone loss (50). Many of these steroid-related side effects clearly improve after withdrawal of prednisone (51), but at the cost of an increased incidence in acute rejection (38). Certainly, in the short-term the risk of graft failure is low. However, concern has been raised by reports relating steroid-withdrawal with long-term allograft failure in patients on cyclosporine-based regimens (38). Newer more potent immunosuppressive drugs, such as tacrolimus (52) and MMF might overcome the problem of acute rejection after steroid-withdrawal. We have evaluated the efficacy of daclizumab as replacement therapy for steroids in renal allograft recipients treated with a combination of tacrolimus and MMF (53). All patients also received prednisolone intravenously for three days after transplantation. Control patients received corticosteroids for only four months after transplantation. The actual benefit of our steroid-free regimen was limited. Cardiovascular risk factors were temporary improved, but at twelve months after transplantation differences no longer existed between the two groups. Besides, the steroid-free regimen tended to increase the severity of the acute rejection episodes, had no important benefit on the rate of bone loss in the first year after transplantation, and was more expensive. Thus, the regimen used in our control group,

consisting of four months of corticosteroid therapy combined with tacrolimus and MMF seems a reasonable option to limit steroid-related side effects after transplantation. It remains to be determined if the steroid-related side effects can be further limited by avoiding the initial i.v. prednisolone bolus. Furthermore it has not been proven that the addition of anti-IL-2Rα therapy is really necessary to achieve the results that were obtained with the steroid-free regimen.

Alternative indications for anti-IL-2Rα therapy in renal transplantation

Anti-rejection therapy

Thus far anti-IL-2Rα therapy has been mainly applied as induction treatment for prevention of acute rejection. Several uncontrolled studies have claimed the usefulness of anti-IL-2Rα therapy in the treatment of acute rejection after organ transplantation. These studies however were confounded by the concomitant conversion of cyclosporine to tacrolimus (54), by the concomitant lowering of the dose of tacrolimus in a patient with tacrolimus toxicity (55), and by the absence of histological proof of the rejection episodes (56). Controlled studies have to be awaited to define the role of IL-2Rα antibodies in the treatment of acute rejection.

Prevention of rejection after conversion of immunosuppressive regimen

After successful transplantation the dose of immunosuppression can be gradually lowered. Many centers in fact try to reduce the intensity of immunosuppression. Well-known examples of such changes of immunosuppression are the conversion of cyclosporine and prednisone to azathioprine and prednisone. However, the replacement of cyclosporine by azathioprine results in the occurrence of an acute rejection in approximately 20% of the patients (37;38). It is unknown if anti-IL-2Rα therapy will reduce this rejection rate. In an uncontrolled study, daclizumab was given during the conversion from tacrolimus- to sirolimus-based immunosuppression (47). In this study only one of 27 patients (3%) experienced an acute rejection. However, in another study no rejection episodes occurred in 15 patients during conversion from tacrolimus- to sirolimus-based immunosuppression without additional anti-IL-2Rα therapy (57). Controlled studies are needed to determine whether anti-IL-2Rα therapy can prevent rejection episodes during conversion of immunosuppressive regimens.

Monitoring of IL-2Rα therapy

We have developed an alternative method for monitoring the presence of daclizumab in serum by measurement of the fractional excretion of soluble IL-2Rα (58;59). This method proved more sensitive than the current gold standard, flow cytometric measurement of IL-2Rα on peripheral lymphocytes. The clinical value of monitoring the level and duration of IL-2Rα blockade has still to be determined. While therapeutic drug monitoring is not necessary in patients treated with a standard dose of anti-IL-2Rα mAb (60), monitoring might be of value to determine the minimum dose of the (expensive) IL-2Rα blocking monoclonal antibodies required for effective rejection prophylaxis. Furthermore, monitoring might become relevant if alternative treatment protocols using anti-IL-2Rα therapy are developed i.e. prolonged treatment in patients with severe toxicity of standard immunosuppressive drugs.

Prevention of (steroid-related) bone loss

Renal allograft recipients are at increased risk to develop bone fractures after transplantation (61). Loss of bone mass after transplantation has been attributed to the use of steroids. Therefore, we have used a nearly steroid-free regimen consisting of daclizumab, tacrolimus, and MMF. Although we cannot exclude that bone loss is attenuated by this regimen, the benefits compared to the control regimen, which only included 4 months of prednisone treatment, were limited (62). However, it is important to note, that bone loss in our control patients was almost negligible, making it very difficult to demonstrate a beneficial effect. In the past years we have regularly measured bone loss in our transplant patients. If we compare bone loss in the various patient cohorts, it becomes obvious that there is a clear relation between loss of bone mass and steroid dose (Figure 1). Furthermore in a controlled trial we have demonstrated that treatment with active vitamin D and calcium attenuates bone loss (63). Other investigators have demonstrated a comparable benefit using biphosphonates (64;65). Thus, there are several treatment options for patients at high risk for bone fractures: choose a regimen with the lowest possible dose of steroids; add active vitamin D and calcium or bisphosphonates. Future studies have to determine whether prophylactic treatment with vitamin D or bisphosphonates is of additional value in renal allograft recipients treated with such a low dose of steroids.

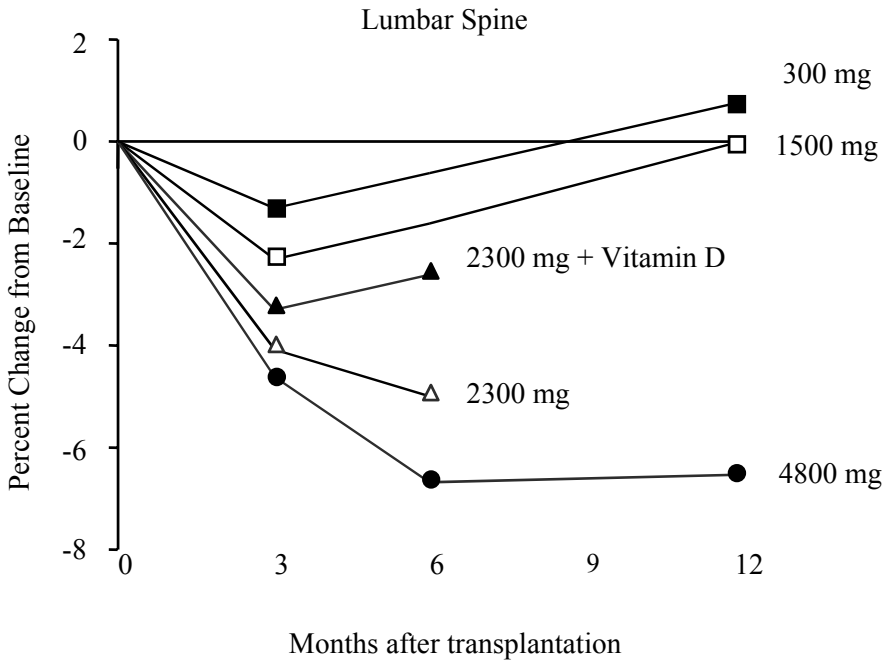

Figure 1. The relation between the use of corticosteroids, and the changes in bone mineral density during the first year after transplantation in studies performed at our center (62;63;66). The median cumulative steroid dose of each group is presented at the end of each line. In one study group additional treatment with vitamin D was given. (black versus open squares: steroid-free regimen versus 4 months steroids (62); closed versus open triangles: Vitamin D and calcium prophylaxis versus no prophylaxis (63); closed circles (66)).

Future perspectives

The ideal solution to prevent immunologic destruction of organ allografts would be to make recipients of a allograft tolerant to donor tissues. After induction of tolerance no immunosuppressive drugs are needed for preservation of allograft function. The role of anti-IL-2Rα therapy in tolerance induction protocols is controversial. For optimal allograft acceptance, allograft-specific recognition and activation of T-cells should occur, leading to the eventual apoptosis of the alloreactive cells. Several experiments indicate that therapies that target the IL-2 pathway such as anti-IL-2Rα therapy and calcineurin inhibitors, prevent the induction of allograft acceptance by hindering apoptosis of these alloreactive cells (67-69).

The most exciting development in induction of (partial) tolerance in human renal transplantation has been reported by the groups of Calne (70) and Startzl (71). In their uncontrolled studies, tolerance to the allograft was induced by a regimen consisting of a pan T-cell depleting antibody followed by a (very) low dose of a calcineurin inhibitor. Steroids were only given before transplantation to prevent cytokine-release that follows the infusion of antilymphocyte antibodies. Graft and patient survival were excellent. Interstitial infiltrates, suggestive for acute rejection were frequently seen in protocol biopsies, but in the absence of graft dysfunction, the authors did not consider these infiltrates as harmful, but as a stage in the evolution of immunological tolerance. Immunosuppression related morbidity was virtually eliminated with these regimens. Although very promising, controlled studies have to be awaited before implementation can be advised.

Another promising area of research is the development of tools to measure the immunological status of the renal allograft recipients. In pilot studies it was shown that the absence of donor-specific cytotoxic T-lymphocytes predicted the successful withdrawal of immunosuppression (72). We must await further development and implementation of these techniques after renal transplantation. With these tools, the degree of immunosuppression can be modified to the individual needs required for optimal rejection prophylaxis.

Conclusion

One-year graft survival is excellent with currently available immunosuppressive drugs. Anti-IL-2Rα therapy decreases the acute rejection rate when added to a calcineurin inhibitor-based immunosuppressive regimen. However, augmenting the immunosuppression with the highly selective acting IL-2Rα antibodies did not improve three-year graft survival. The lack of toxicity of anti-IL-2Rα therapy has been the rationale to replace other, more toxic

immunosuppressive drugs by anti-IL-2Rα therapy. Replacement of corticosteroids after transplantation by administration of the IL-2Rα antibody daclizumab is feasible. However, the benefit of this expensive regimen is limited compared with a regimen with limited steroid exposure. Anti-IL-2Rα therapy is not efficacious enough to replace the continuous use of a calcineurin inhibitor after renal transplantation. Sirolimus on the other hand, seems efficacious enough to replace the continuous use of calcineurin inhibitor. It is unclear whether anti-IL-2Rα therapy is of additional value to a sirolimus-based immunosuppressive regimen. Considering the excellent one-year graft survival achieved with the current immunosuppressive regimens, long-term follow-up is required to detect potential benefits of these newly developed regimens.

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Curriculum vitae

Rik ter Meulen werd 9 april 1966 geboren in Sittard. In 1984 behaalde hij het Atheneum B diploma aan het Rijksscholengemeenschap F.A. Minkema te Woerden en in 1992 het artsexamen aan de Vrije Universiteit te Amsterdam. De opleiding tot internist werd gestart in het Catharina Ziekenhuis te Eindhoven (1993-1997; opleiders: prof. dr. H.F.P. Hillen, dr. W.P.M. Breed en dr. S.J. Hoorntje) en werd voortgezet in het Radboud Ziekenhuis te Nijmegen (1997-1999; opleider: prof. dr. J.W.M van der Meer). In april 1999 vond registratie plaats als internist. Erna werd de opleiding in het aandachtsgebied nefrologie gevolgd (opleider: prof. dr. R.A.P. Koene). Registratie als internist-nefroloog vond plaats in oktober 2000. Erna was hij werkzaam als junior staflid op de afdeling Nierziekten in het Radboud Ziekenhuis. In december 1999 startte hij met het in dit proefschrift beschreven onderzoek. Per 1 oktober 2003 is hij als internist-nefroloog toegetreden tot de maatschap Interne Geneeskunde in het Canisius-Wilhelmina Ziekenhuis te Nijmegen. Hij is getrouwd met Akosua de Groot. Samen hebben zij drie kinderen, Daan, Jaap en Margot.