Nephrol Dial Transplant (2007) 23: 1422–1428
doi: 10.1093/ndt/gfm788
Advance Access publication 18 December 2007

Original Article

NDT
Nephrology Dialysis Transplantation

Glucocorticoid resistance in dialysis patients may impair the kidney allograft outcome

Sergio R. De Antonio, Luciana T. S. Saber, Rosangela S. Chriguer and Margaret de Castro

Department of Internal Medicine, School of Medicine of Ribeirao Preto, University of Sao Paulo, Avenida dos Bandeirantes, 3900, 14049-900, Ribeirao Preto, SP, Brazil

Abstract

This study examines *in vitro* steroid sensitivity in chronic renal failure (CRF) patients and its influence on the allograft outcome. We determined the inhibitory effect of dexamethasone (DEX) on concanavalin A (Con-A)-stimulated peripheral blood mononuclear cell (PBMC) proliferation, and glucocorticoid receptor' (GR) number of binding sites (B_{max}) and affinity (K_d) in 28 CRF patients and 40 normal healthy controls. Based on K_d values >95th percentile from controls, patients were divided into two groups: glucocorticoid resistant ($n = 11$) and glucocorticoid sensitive ($n = 17$). Patients were followed during 18 months post-transplantation observing acute rejection episodes (ARE), chronic allograft nephropathy (CAN), allograft failure and death. The DEX concentration that caused 50% inhibition of Con-A-stimulated PBMC proliferation (IC_{50}) was higher in CRF than in healthy controls ($2.2 \times 10^{-5} \pm 1.0 \times 10^{-5}$ versus $8.3 \times 10^{-6} \pm 4.2 \times 10^{-6}$ mol/L, $P = 0.02$). Values of K_d (12.4 ± 1.8 versus 7.2 ± 0.9 nM) and B_{max} (7.7 ± 1.1 versus 4.1 ± 0.3 fmol/mg protein) were higher in CRF patients ($P = 0.02$ and $P = 0.001$, respectively). There were higher incidences of ARE ($P = 0.02$) and CAN ($P = 0.002$) in the glucocorticoid-resistant group. Univariate and multivariate logistic regression showed that K_d was an independent predictor of ARE (OR 8.8, $P = 0.03$) as well as of CAN (OR 16.5, $P = 0.01$). In conclusion, we observed glucocorticoid resistance in a subgroup of CRF patients undergoing dialysis, which led to a higher morbidity due to ARE and CAN in an 18-month follow-up period.

Introduction

Glucocorticoids play an important role in the endocrine control of homeostasis, immune functions, cell growth and differentiation and cell death, and are often used to treat different immune-mediated diseases, e.g. autoimmune diseases, rheumatological diseases, kidney disorders and organ transplantation [1–3]. Chronic renal failure (CRF) has been described as a chronic systemic inflammatory disease. Several mechanisms are likely to contribute to the activation of the inflammatory response in CRF, which can lead to glucocorticoid resistance. Among these are reduced renal clearance of proinflammatory mediators [tumour necrosis factor alpha, interleukin (IL)-6], accumulation of advanced glycoxidation end products, production of reactive oxygen species and oxidative damage and chronic infection [4].

The powerful effects of glucocorticoids have justified their use in almost all drug combinations in kidney transplantation to maintain immunosuppressive therapy and minimize the incidence of acute rejection episodes (ARE) optimizing the first-year renal graft survival [5,6]. Despite these advances, chronic allograft nephropathy (CAN), the leading cause of transplant failure in long-term follow-up, remains elevated and without adequate treatment. The incidence of CAN is associated with delayed graft function, HLA matching, cytomegalovirus-associated infection, timing of transplantation, donor and recipient age and race, recipient sensitization, acute rejection and immunosuppression regimen [7]. Therefore, a satisfactory outcome of the renal transplantation depends upon adequate control of the immune system.

Glucocorticoids interact with the cytoplasmic glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily [8]. The magnitude of cell response to glucocorticoids depends on the ability of the cell to receive and transduce the hormonal signal, i.e. on the presence of GR in an adequate number, as well as on the efficiency of receptor-mediated signal transduction [8].

Previous studies have demonstrated an increased incidence of lymphocyte resistance to the effects of glucocorticoids in peripheral blood mononuclear cells (PBMC) from CRF patients awaiting renal transplantation and have correlated this resistance with the poor outcome of the renal graft [9,10]. In addition, decreased ability of PBMC to amplify GR numbers in response to a mitogenic stimulus in culture medium may contribute to glucocorticoid resistance in PBMC of CRF patients [11]. Studies correlating GR-binding alterations in fresh PBMC from CRF patients undergoing dialysis with the allograft outcome are lacking. The binding assay is more straightforward than the

Correspondence and offprint requests to: Margaret de Castro, School of Medicine of Ribeirao Preto, University of Sao Paulo, Avenida dos Bandeirantes, 3900, 14049-900, Ribeirao Preto, SP, Brazil. Tel: +55-16-3602-2940; Fax: +55-16-3633-6695; E-mail: castrom@fmrp.usp.br

glucocorticoid-mediated inhibition of mitogen-stimulated PBMC proliferation. Therefore, we hypothesized whether GR indices could be of clinical significance for the prediction of the allograft outcome in kidney transplantation.

Material and methods

Subjects

This prospective study was approved by the Institutional Review Board for Human Research at the Clinical Hospital of the School of Medicine of Ribeirao Preto—University of Sao Paulo (Proc HCRP no 4522/2003) and informed consent was obtained from all subjects.

This study included 28 chronic renal failure patients, 19 males and 9 females, ranging in age from 18 to 64 years. All CRF patients admitted to renal transplantation, from February 2003 to July 2004, in the Transplant Unity of the University Hospital of the School of Medicine of Ribeirao Preto—University of Sao Paulo were eligible to be included in this study. The exclusion criteria were previous use of immunomodulatory drugs, including glucocorticoids, for at least 3 months before the study and patients who would be submitted to kidney transplantation after 22:00 h due to technical difficulties. In addition, 40 healthy control subjects (19 males and 21 females, ranging in age from 22 to 42 years) with no history of acute or chronic illness or use of any medication for at least 3 months were also previously evaluated in our laboratory [12]. Clinical characteristics such as race, age, sex, aetiology of CRF, type of kidney donor, cold-ischaemia time, number of HLA mismatches, serum levels of panel-reactive antibodies (PRA) and the regimen of initial immunosuppressive therapy were evaluated.

PBMC isolation

Blood samples were obtained from CRF patients at the time they were submitted to renal transplantation, immediately before receiving induction immunosuppressive therapy. PBMC were isolated by density-gradient centrifugation using Ficoll–Hypaque (Histopaque; Sigma Chemical Co., St Louis, MO, USA), washed three times in Hank's buffered saline solution (HBSS) and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 2 mmol/L HEPES buffer (Sigma Chemical Co.), 10% fetal calf serum, 100 IU/mL penicillin, 100 g/mL streptomycin and 10 mg/mL gentamicin.

Proliferation and in vitro glucocorticoid sensitivity assay

To perform the *in vitro* steroid sensitivity assay, we determined the inhibitory effect of dexamethasone (DEX) on concanavalin A (Con-A)-stimulated PBMC proliferation, as previously described [12,13]. PBMC (2×10^6 cells per well) were plated onto 96-well flat-bottomed plates (Nunc, Denmark) in triplicate and cultured at 37°C in the presence of 5% CO_2. Con-A at a dose of 50 μg/mL was used to stimulate the cells in the presence or absence of different doses (10^{-8}, 10^{-6} and 10^{-4} mol/L) of DEX. After 48 h of culture, the cells were pulsed with 1 μCi/well-tritiated thymidine (^{3}H-thymidine; Armersham, Pharmacia Biotech, UK) for 18 h. The cells were harvested with a multiple automated sample harvester and radioactivity was counted in a liquid scintillation β counter (Beckman Coulter, Fullerton, CA, USA).

Binding assay

PBMC DEX-binding assay was performed as previously described [12,13]. Cells were suspended in RPMI media and adjusted to 2×10^6 cells per tube in duplicate and incubated with six concentrations (1.56–50 nmol/L) of DEX (1, 2, 4, 6, 7, ^{3}H) (Dexamethasone; Amersham Life Science, Buckinghamshire, UK) at 37°C in the presence or absence of a 1000-fold molar excess of unlabelled DEX (Dexamethasone; Sigma Chemical Co.) for 1 h. After incubation, the cells were washed three times to separate bound from free steroid with 1.5 mL cold phosphate-buffered saline (PBS) and centrifuged at 400 *g* for 10 min. After the third wash, the pellets were suspended in 100 μL of RPMI, transferred to vials and radioactivity was counted in a β counter. All values obtained were corrected for nonsaturable binding for each respective concentration. Saturation binding analysis was performed assuming a linear binding plot of the bound divided by the free-tritiated DEX concentration to the amount bound at an infinite free-hormone concentration. Receptor sites per cell (B_{max}) and the dissociation constant (K_d) were calculated by the method of Scatchard using computerized squares linear regression. The B_{max} (GR-binding capacity) was expressed as fmol of DEX bound per mg of protein, and K_d (GR binding-affinity), which is inversely proportional to ligand affinity, was expressed in nmol/L. Protein content was measured using BCA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as standard.

Post-transplant clinical follow-up

After renal transplantation, all patients were followed for at least 18 months by the Medical Staff of the Transplant Unit of the University Hospital of School of Medicine of Ribeirao Preto—University of Sao Paulo. To evaluate renal allograft outcome during follow-up, we divided the patients into two groups: glucocorticoid sensitive and glucocorticoid resistant, based on the K_d values. To identify glucocorticoid-resistant patients, we used the 95th percentile of the K_d values from those 40 healthy subjects previously studied [12]. Allograft failure was defined by the need for long-term dialysis after transplantation, repeated transplantation or death. All ARE were biopsy proven. CAN was confirmed by biopsies performed under clinical indication based on biological marker abnormalities of graft function (serum creatinine, urinary sediment and Cockroft- and Gault-estimated creatinine clearance). All biopsies were analysed by the same blinded pathologist, and ARE and CAN were diagnosed according to Banff criteria [14].

Data analysis and statistics

All results are expressed as mean ± SEM and percentiles, when appropriate. Statistics were carried out using the nonparametric Mann–Whitney test for continuous variables or

Fisher's exact test for categorical data. For the analysis of DEX-mediated inhibition of Con-A-stimulated PBMC proliferation, the IC_{50} was defined as the concentration of DEX that caused 50% inhibition of cell proliferation. To obtain more estimated individual values, the *in vitro* data were adjusted to a nonlinear mixed logistic growth model with fixed and random effects [12,13] based on the equation $y_{ij} = (b_1 + \mu_{i1}/1 + \exp[-(d_{ij} - b_2)/b_3]) + \varepsilon_{ij}$, where y_{ij} is the percentage of inhibition of PBMC in a j dose of DEX in an i^{h} observation, d_{ij} represents the doses of DEX, b_1 represents the asymptotic regression, b_2 the point of inflection and b_3 the parameter form, μ_{i1} represents individual residual effects and ε_{ij} residual errors. For analysis and data simulation, we used the PROC NL MIXED software SAS version 8.02 (SAS Institute, Cary, NC, USA). Patients with IC_{50} values higher than the 95$^{\text{th}}$ percentile (P95) of the normal subjects were considered resistant to glucocorticoid.

We used the Spearman correlation to compare the individual IC_{50} obtained from DEX inhibition of Con-A-stimulated PBMC proliferation with the K_{d} values obtained from binding assay of the same subject. We also used the Spearman correlation to compare the individual K_{d} and $B_{\max}$ values. Receiver operating characteristic (ROC) curves were generated to detect the best K_{d} values associated with ARE and CAN. Uni- and multivariate logistic regressions were performed to exclude confounding factors. The nonparametric area under the ROC curve and 95% confidence intervals and logistic regression were calculated using the Statistical Package for the Social Sciences, version 10 (SPSS inc., Chicago, IL, USA). Survivorship curves were evaluated using the Kaplan–Meier technique and were compared using the Wilcoxon test. Significance was assumed when $P < 0.05$.

Results

Among 28 CRF patients, 26 were undergoing haemodialysis three times a week for 4 h using low-flux polysulfone membranes and 2 were undergoing continuous ambulatory peritoneal dialysis. The period of dialysis was 50 ± 6.3 months. The aetiology of CRF was unknown in 28.5%; diabetes mellitus in 17.9%; hypertension in 17.9%; urologic in 14.3%, polycystic kidney disease in 10.7% and glomerulonephritis in 10.7%. Three patients received a kidney transplant from a living related donor and 25 from deceased donors. In the deceased donor transplants, the kidney cold ischemia time ranged from 18 to 46 h (32.9 ± 1.5 h). The number of HLA mismatches was 3.1 ± 0.13. The serum levels of PRA were: PRA >40% in 3 patients (10.7%); PRA = 20% in 1 patient (3.5%); PRA = 10% in 6 patients (21.5%) and undetectable levels of PRA in 18 patients (64.3%).

Regarding immunosuppressive regimen, all patients received 1 g of methylprednisolone I.V. at the time of transplantation. The following maintenance regimen consisted of prednisone, initially at 1 mg/kg/day followed by a gradual reduction (0.5 mg/kg/day for 4 weeks, 0.2 mg/kg/day for 8 weeks, 0.15 mg/kg/dia for 6 months and 0.05 to 0.10 mg/kg/day after the first year) in combination with tacrolimus and micofenolate mofetil in 12 patients; cyclosporine A and micofenolate mofetil in 8 patients; cyclosporine A and azathioprine in 4 patients; tacrolimus and azathioprine in 2 patients and sirolimus and micofenolate mofetil in 2 patients. Twenty patients (71.4%) received an anti-IL-2 receptor antibody associated with the initial immunosuppressive regimen, when PRA was >30% or cold ischemia time >24 h.

Proliferation assay

The inhibitory effect of DEX on Con-A-stimulated PBMC proliferation was performed on 18 out of 26 CRF patients. Basal lymphocyte proliferation was stimulated by Con-A in the CRF group (1826 ± 812 versus 31140 ± 5735 c.p.m.). Increasing concentrations of DEX (10^{-8}, 10^{-6} and 10^{-4} mol/L) inhibited lymphocyte proliferation in a dose-dependent manner (19270 ± 2930; 12080 ± 2578; 12110 ± 2485 c.p.m.).

The individual IC_{50} values estimated by the PROC NL MIXED software for *in vitro* glucocorticoid sensitivity analysis showed that as a group, the mean (± SEM) IC_{50} value was higher ($P = 0.02$) in the CRF group: $2.2 \times 10^{-5} \pm 1.0 \times 10^{-5}$ (range: 1.3×10^{-8} to 1×10^{-4} mol/L) than in the control group: $8.3 \times 10^{-6} \pm 4.2 \times 10^{-6}$ mol/L (range: 5.0×10^{-8} to 1×10^{-4} mol/L). The IC_{50} 95th percentile value of the control group was 4.4×10^{-6} mol/L. According to this cut-off value, four CRF subjects were considered resistant to glucocorticoid; these patients did not fit to the logistic model indicating that the DEX dose necessary to suppress 50% of PBMC proliferation in these individuals was higher than 10^{-4} mol/L. For the purpose of statistical analysis, in these four individuals, we considered the highest dose used in the experiment (1×10^{-4} mol/L).

Binding assay

The linearity of the Scatchard plots indicates a single class of binding site affinity. The mean ± SEM of the number of binding sites of GR ($B_{\max}$) in PBMC and their dissociation constant (K_{d}) were 4.1 ± 0.3 fmol/mg of protein and 7.2 ± 0.9 nmol/L in healthy subjects, and 7.7 ± 1.1 fmol/mg of protein and 12.4 ± 1.8 nmol/L in the CRF group. As a group, CRF patients had $B_{\max}$ and K_{d} values significantly higher than those of healthy subjects ($P = 0.001$ and $P = 0.02$, respectively). Figure 1 shows a representative saturation curve of two CRF patients, one with normal K_{d} and the other with an elevated K_{d}. Considering the values above the 95th percentile of the normal group ($B_{\max} > 7.9$ fmol/mg of protein and $K_{\text{d}} > 9.1$ nmol/L), 1 out of 40 controls (2.5%) and 9 out of 28 CRF patients (32.1%) showed an elevated $B_{\max}$ ($P = 0.0006$), while 4 out of 40 controls (10%) and 11 out of 28 CRF patients (39%) showed an elevated K_{d} ($P = 0.02$). There was a positive correlation between individual K_{d} and $B_{\max}$ values in the CRF group ($r = 0.45$; $P = 0.01$).

Correlation between the in vitro assays

Eighteen out of 26 CRF patients were studied by both proliferation and binding tests. The individual IC_{50} obtained from DEX inhibition of Con-A-stimulated PBMC proliferation

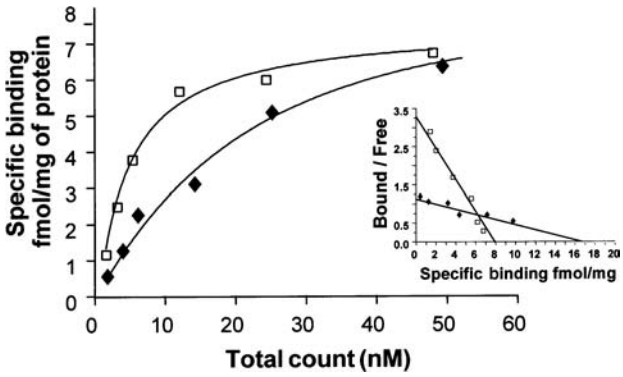

Fig. 1. Glucocorticoid receptor-binding abnormalities in chronic renal failure patients. Representative saturation curves of [^{3}H] dexamethasone binding to peripheral blood mononuclear cells and the Scatchard plot (inset) of glucocorticoid receptor-binding studies of one individual with normal K_d (□) and of one individual with an elevated K_d from the CRF group (♦).

did not correlate with the K_d or B_{max} values obtained from binding assay of the same subject. We found a concordance between K_d values and IC_{50} obtained from the proliferation test in only 11/18 CRF patients (61%) compared to 36/40 control subjects (90%) observed in a previous study [12]. In the CRF patients, among the nine patients who showed elevated K_d, three needed the highest DEX dose (10^{-4} mol/L) to inhibit at least 50% or showed no inhibition of Con-A-stimulated proliferation. However, it is important to point out that among the six patients with elevated K_d and IC_{50} below the 95th percentile of the normal group, five showed elevated B_{max}.

The ligand affinity parameter (K_d) was considered the primary mechanism of glucocorticoid resistance in CRF patients and the elevation of glucocorticoid-binding sites only a compensatory mechanism. Therefore, in the present study, we chose the ligand affinity parameter (K_d) instead of GR-binding capacity (B_{max}) as the criterion for glucocorticoid resistance. Then, based on the 95th percentile of the K_d values of the normal subjects, CRF patients were divided into glucocorticoid-sensitive ($n = 17$) and glucocorticoid-resistant ($n = 11$) groups. Table 1 shows clinical and laboratorial findings for both groups. There was no difference between glucocorticoid-sensitive and glucocorticoid-resistant patients in any clinical or biochemical characteristics, including the glomerular filtration rate (GFR, ml/min/1.73 m^2) at 1 month (39.9 ± 18.4 versus 39.4 ± 19.8, $P = 0.9$), 6 months (51.2 ± 23.4 versus 47.4 ± 14.0, $P = 0.7$), 12 months (53.6 ± 18.1 versus 41.0 ± 18.8, $P = 0.3$) and 18 months (56.1 ± 22.8 versus 40.4 ± 12.1, $P = 0.2$) after kidney transplantation. No differences were noted between the groups regarding glucocorticoid dose or maintenance of immunosuppression regimen.

Allograft outcome

After renal transplantation, all patients were followed for at least 18 months and the occurrence of acute rejection, CAN, allograft loss by any cause and death was determined. In the present study, no correlation between PBMC resistance to DEX in the Con-A-stimulated proliferation assay before transplant and clinical outcome of the recipients post-transplant was observed. However, there was a higher incidence of acute rejection in CRF glucocorticoid-resistant (elevated K_d) compared to CRF glucocorticoid-sensitive patients (55.6% versus 12.5%, $P = 0.02$). Acute rejection was treated with the same dose of intravenous methylprednisolone in glucocorticoid-resistant or -sensitive groups. There was also a higher incidence of CAN in glucocorticoid-resistant compared to glucocorticoid-sensitive patients (75% versus 15.4%, $P = 0.002$) (Figure 2). Allograft failure and number of deaths at 18 months of follow-up were not significantly different between both groups (Figure 3). The ROC curves evaluated the ability of K_d to discriminate between the presence and absence of ARE and CAN (Figure 4). When a K_d cut-off value of 10.5 nM was applied, the sensitivity and specificity of K_d to identify glucocorticoid-sensitive and glucocorticoid-resistant patients with acute rejection were both 71.4% and with chronic allograft nephropathy were both 75%.

Univariate logistic regression showed that K_d was a predictor of acute rejection (OR 8.8, 95% CI 1.2–63.4; $P = 0.03$) as well as of CAN (OR 16.5, 95% CI 1.8–148.6; $P = 0.01$). In addition, acute rejection was also a predictor of CAN (OR 20, 95% CI 1.7–241.7; $P = 0.02$). B_{max}, PRA, number of mismatches, cold ischemia time and acute tubular necrosis were not predictor factors of acute rejection or CAN.

Multivariate logistic regression showed that K_d was an independent predictor of acute rejection (OR 18.9, 95% CI 1.3–269; $P = 0.03$) when analysed with panel >30, cold ischemia time >24 h and acute tubular necrosis, and showed a trend of being an independent predictor of acute rejection (OR 15.9, 95% CI 0.9–308; $P = 0.06$) when the number of mismatches >3 was added in the multivariate analysis. K_d and acute rejection remained independent predictors of CAN (OR 14.8, 95% CI 1.1–205.0 and OR 17.8, 95% CI 1.0–340.0, respectively, $P = 0.05$) by multivariate logistic regression.

Discussion

Glucocorticoid resistance has been extensively studied in patients with idiopathic nephrotic syndrome [13], asthma [15], rheumatoid arthritis [2], familial cortisol resistance [16] and also in normal subjects [12]. In the present study, we observed that CRF patients undergoing dialysis showed an increased number and a decreased affinity of GR to DEX compared to healthy subjects, suggesting glucocorticoid resistance in uraemia. In addition, we demonstrated that glucocorticoid resistance negatively influenced the 18-month kidney transplant outcome.

Using a logistic mathematical model, we observed that some CRF patients showed no inhibition or needed high DEX doses to inhibit 50% of the Con-A- stimulated lymphocyte proliferation suggesting glucocorticoid resistance in a subset of CRF patients, in accordance to previous reports using a similar methodological approach [9,10]. It has also been demonstrated that patients with lymphocyte resistance to glucocorticoids evaluated by proliferation assays

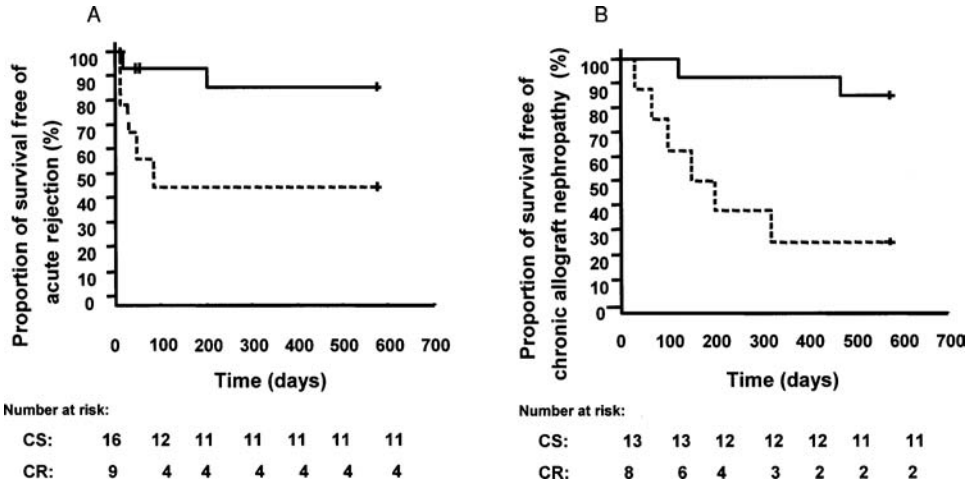

Fig. 2. Kaplan–Meier curves of cumulative proportions. (**A**) Survival free of acute rejection ($P = 0.02$) and (**B**) survival free of chronic allograft nephropathy ($P = 0.002$). The continuous line (–) represents the glucocorticoid–sensitive group and the dotted line (- -) represents the glucocorticoid-resistant group. *P*: for the comparison between the groups.

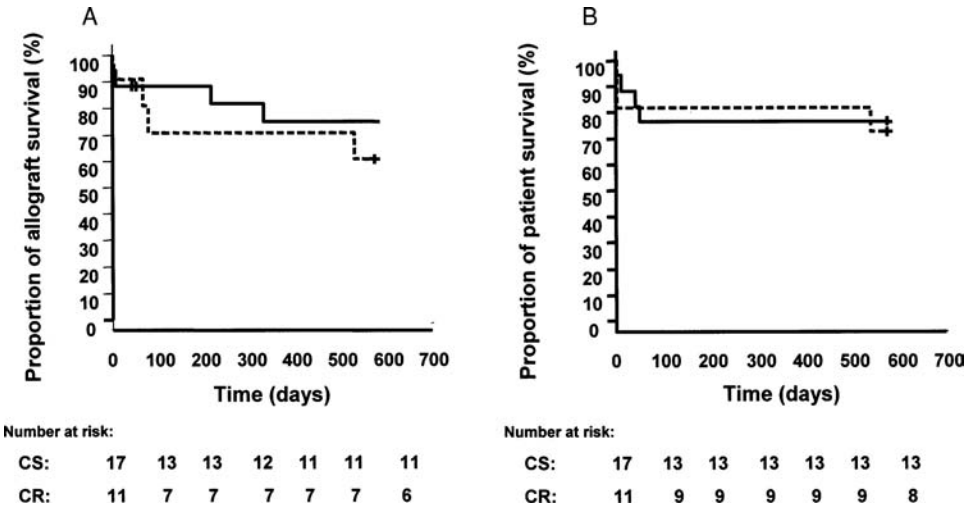

Fig. 3. Kaplan–Meier curves of cumulative proportions. (**A**) Survival of kidney allograft (NS); and (**B**) patient survival (NS). The continuous line (–) represents the glucocorticoid–sensitive group and the dotted line (- -) represents the glucocorticoid–resistant group. *P*: for the comparison between groups.

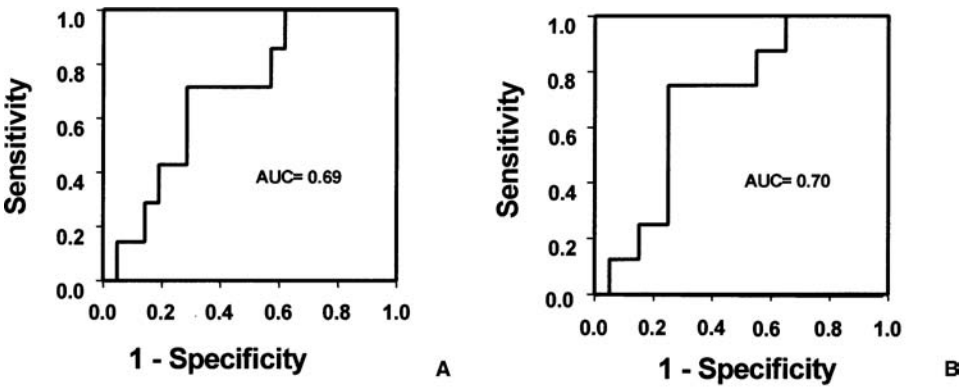

Fig. 4. Receiver–operating characteristic (ROC) curves showing the diagnostic performance of K_d in predicting acute rejection episodes (**A**) and chronic allograft nephropathy (**B**). K_d cut-off value of 10.5 nM shows sensitivity and specificity of 71.4% and 75% to predict acute rejection and chronic allograft nephropathy, respectively. AUC = area under the curve.

Table 1. Clinical and laboratorial findings of corticosteroid–sensitive (CS, $n = 17$) and corticosteroid–resistant (CR, $n = 11$) patients with chronic renal failure at the time of transplantation

Characteristic	CS group ($n = 17$)	CR group ($n = 11$)	P
Race of recipient (%)			
White	11 (64.7)	6 (54.5)	0.7
Black	4 (23.5)	2 (18.2)	1.0
Other	2 (11.8)	3 (27.3)	0.3
Sex of recipient (% male)	13 (76.5)	6 (54.5)	0.4
Age of recipient (years)	44 ± 3.3	43.3 ± 3.4	0.8
Cause of native kidney disease (%)			
Diabetes mellitus	5 (29.4)	0 (0)	0.1
Hypertension	3 (17.6)	2 (18.2)	1.0
Urologic disease	2 (11.8)	2 (18.2)	1.0
Polycystic kidney disease	1 (5.9)	2 (18.2)	0.5
Glomerulonephritis	2 (11.8)	1 (9.1)	1.0
Other or unknown	4 (23.5)	4 (36.3)	0.7
Duration of dialysis (months)	47.9 ± 8.0	53.5 ± 10.6	0.7
Most recently determined serum levels of panel-reactive antibodies (% > 10%)	11.0	18.0	1.0
Deceased donor (%)	16 (94.1)	9 (81.9)	0.5
Cold ischemia time (h)	32.2 ± 1.5	34.2 ± 3.3	0.4
No. of HLA mismatches	3.1 ± 0.23	3.0 ± 0.0	1.0
Initial immunosuppression (%)			
Corticosteroids	100	100	1.0
Cyclosporine or FK 506	94	91	1.0
Mycophenolate or azathioprine	100	100	1.0
Sirolimus	6	9	1.0
Anti-IL-2 antibodies	70	72	1.0

Values are means ± SEM.

showed a high risk of acute allograft rejection or graft loss under glucocorticoid therapy [17,18]. In the present study, no correlation between PBMC resistance to DEX in the Con-A-stimulated proliferation assay before transplant, and clinical outcome of the recipients post-transplant, was observed.

Glucocorticoid resistance, due to GR-binding abnormalities, has been observed in about 2 to 10% of the normal controls, suggesting that GR might mediate glucocorticoid resistance even in some healthy subjects [12]. There are few reports concerning glucocorticoid resistance in CRF using a binding assay. Therefore, in order to better understand the mechanisms underlying the glucocorticoid resistance in patients with CRF undergoing dialysis, the number and affinity of GR were also studied. As a group, GR affinity and capacity abnormalities were more frequently observed in CRF patients compared to normal subjects. Nine out of 11 CRF patients who presented elevated K_d were also studied by proliferation assay. Among these patients, three showed no inhibition or needed the highest DEX dose (10^{-4} mol/L) to inhibit 50% of Con-A-stimulated lymphocyte proliferation and six patients inhibited Con-A-stimulated proliferation with more physiological DEX doses ($<10^{-6}$ mol/L). However, five out of these six patients showed elevated B_{max}, suggesting a compensatory increase in the number of glucocorticoid binding sites in order to overcome the GR resistance.

Our data clearly demonstrated that a subgroup of CRF patients undergoing dialysis presented a decreased affinity of GR to DEX suggesting glucocorticoid resistance in uraemia. It is important to point out that, besides abnormalities in the number and affinity of GR α, we cannot rule out an overexpression of GR β, a dominant negative inhibitor of GR α, inducing steroid insensitivity in CRF, as well established in glucocorticoid- resistant patients with asthma, rheumatoid arthritis and ulcerative colitis [19]. In addition, polymorphisms in the GR gene and other factors involved in the multiple GR signal transduction pathways, such as glucocorticoid interaction with heat-shock protein and with co-activator or co-repressor proteins, and also GR communication with different transcription factors might mediate the variable response to glucocorticoids. Finally, another possible mechanism of glucocorticoid resistance in CRF includes an increased production of cytokines, as previously described in glucocorticoid-resistant asthma [15], in glucocorticoid-resistant idiopathic nephrotic syndrome [13] and in rheumatoid arthritis [2]. A similar phenomenon could also occur in the presence of pro-inflammatory cytokine profile described in patients with uraemia [20].

To address the question whether abnormalities in the GR prospectively correlate with the kidney transplant outcome, glucocorticoid-sensitive and glucocorticoid- resistant CRF subgroups were observed for 18 months after transplantation. Regarding acute rejection and CAN, the post-transplant outcomes were not determined according to a fixed protocol since biopsies were performed only under clinical indication; thus, there may be an ascertainment bias. However, in symptomatic patients there was a higher incidence of acute rejection as well as a higher incidence of chronic allograft nephropathy in the glucocorticoid-resistant compared to the glucocorticoid-sensitive patients.

Ribarac-Stepic *et al.* (2001), retrospectively, studied the GR expression in lymphocytes of kidney-transplanted patients and the presence of CAN. The authors observed a

correlation between CAN and reduced number and affinity of GR [21]. However, in contrast to our prospective study in which none of the patients had taken glucocorticoids for at least 3 months prior to the evaluation, those patients were taking glucocorticoid at the time of the study. This issue is relevant since several studies have indicated that glucocorticoid treatment may decrease GR levels in lymphocytes [8] as well as GR mRNA levels [8,22,23] due to homologous down-regulation of hormone receptors by cognate ligands.

K_d was a reliable and independent predictive index for ARE and also for CAN. The area under the ROC curves of 0.69 and 0.70 indicated a fair diagnostic accuracy of K_d to identify acute rejection and CAN, respectively, in glucocorticoid-resistant CRF patients. The best operating point obtained by receiver-operating analysis was K_d of 10.5 nM (sensitivity and specificity of 71.4% for acute rejection, and sensitivity and specificity of 75% for CAN). The K_d cut-off value obtained from the ROC curve was close to the 95th percentile of the K_d value of the normal group (9.1 nM). These data indicate that K_d values above the 95th percentile of the normal population obtained prior to the kidney transplantation may have clinical significance in the kidney transplant outcome. Since there are no previous studies correlating prospectively reduced GR affinity in dialysis patients and its negative influence on allograft outcome, our findings, although observed in a small population, raise an interesting path for future studies.

In conclusion, we observed glucocorticoid resistance in a subgroup of CRF patients undergoing dialysis, which led to a higher morbidity due to ARE and CAN, in an 18-month follow-up period. These data might contribute to choosing alternative immunosuppressive regimens for the subset of patients at greater risks of transplant failure.

Acknowledgements. We thank Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) grant no 03/09857-0. We also thank Professor Roberto Silva Costa for the analysis of kidney biopsies and all professionals of the Transplant Unity of the University Hospital of School of Medicine of Ribeirao Preto and also of the National Center of Organ Sharing -Unity 2 (CNCDO-2). Thanks also goes to Vaughn Christopher Wheelwright for English revision.

Conflict of interest statement. None declared.

References

1. Wilckens T, De Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today* 1997; 18: 418–424
2. De Antonio SR, Blotta HM, Mamoni RL *et al*. Effects of dexamethasone on lymphocyte proliferation and cytokine production in rheumatoid arthritis. *J Rheumatol* 2002; 29: 46–51
3. Morand E. Corticosteroids in the treatment of rheumatologic diseases. *Curr Opin Rheumatol* 1997; 9: 200–205
4. Amore A, Coppo R. Immunological basis of inflammation in dialysis. *Nephrol Dial Transplant* 2002; 17(Suppl 8): 16–24
5. Regazzi MB, Alessiani M, Rinaldi M. New strategies in immunosuppression.*Transplant Proc* 2005; 37: 2675–2678
6. Vincenti F. Immunosuppression minimization: current and future trends in transplant immunosuppression. *J Am Soc Nephrol* 2003; 14: 1940–1948
7. Nankivell BJ, Borrows RJ, Fung CL *et al*. The natural history of chronic allograft nephropathy. *N Engl J Med* 2003; 349: 2326–2333
8. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 1996; 17: 245–261
9. Hirano T, Oka K, Sakurai E *et al*. Impaired prednisolone sensitivities of the endocrine system and peripheral-blood lymphocytes are closely related to clinical incidence in renal transplantation. *J Pharm Pharmacol* 1991; 43: 569–573
10. Kang XX, Hirano T, Oka K *et al*. Role of altered prednisolone-specific lymphocyte sensitivity in chronic renal failure as a pharmacodynamic marker of acute allograft rejection after kidney transplantation. *Eur J Clin Pharmacol* 1991; 41: 417–423
11. Hirano T, Horigome A, Oka K *et al*. Glucocorticoid-resistance in peripheral-blood lymphocytes does not correlate with number of affinity of glucocorticoid receptors in chronic renal failure patients. *Immunopharmacology* 1997; 36: 57–67
12. Chriguer RS, Elias LL, da Silva IM Jr *et al*. Glucocorticoid sensitivity in young healthy individuals: *in vitro* and *in vivo* studies. *J Clin Endocrinol Metab* 2005; 90: 5978–5984
13. Carlotti AP, Franco PB, Elias LL *et al*. Glucocorticoid receptors, *in vitro* steroid sensitivity, and cytokine secretion in idiopathic nephrotic syndrome. *Kidney Int* 2004; 65: 403–408
14. Solez K, Axelsen RA, Benediktsson H *et al*. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int* 1993; 44: 411–422
15. Sher ER, Leung DY, Surs W *et al*. Steroid-resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. *J Clin Invest* 1994; 93: 33–39
16. Mendonca BB, Leite MV, de Castro M *et al*. Female pseudohermaphroditism caused by a novel homozygous missense mutation of the GR gene. *J Clin Endocrinol Metab* 2002; 87: 1805–1809
17. Langhoff E, Ladefoged J, Jakobsen BK *et al*. Recipient lymphocyte sensitivity to methylprednisolone affects cadaver kidney graft survival. *Lancet* 1986; 1: 1296–1297
18. Dumble LJ, Macdonald IM, Kincaid-Smith P *et al*. Correlation between ADCC resistance to *in vitro* steroid and renal allograft failure. *Transplant Proc* 1981; 13: 1569–1571
19. Goecke A, Guerrero J. Glucocorticoid receptor beta in acute and chronic inflammatory conditions: clinical implications. *Immunobiology* 2006; 211: 85–96
20. Jacobs P, Glorieux G, Vanholder R. Interleukin/cytokine profiles in haemodialysis and in continuous peritoneal dialysis. *Nephrol Dial Transplant* 2004; 19 (Suppl 5): V41–V45
21. Ribarac-Stepic N, Isenovic E, Naumovic R *et al*. Glucocorticoid receptors in lymphocytes and stability of kidney graft function. *Clin Exp Med* 2001; 1: 179–186
22. Burnstein KL, Jewell CM, Sar M *et al*. Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. *Mol Endocrinol* 1994; 8: 1764–1773
23. Andreae J, Tripmacher R, Weltrich R *et al*. Effect of glucocorticoid therapy on glucocorticoid receptors in children with autoimmune diseases. *Pediatr Res* 2001; 49: 130–135
24. Grasso G, Lodi L, Lupo C *et al*. Glucocorticoid receptors in human peripheral blood mononuclear cells in relation to age and to sport activity. *Life Sci* 1997; 61: 301–308

Received for publication: 3.7.07
Accepted in revised form: 9.10.07