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RENAL TRANSPLANT PATIENTS MONITORED BY THE CELL-MEDIATED LYMPHOLYSIS ASSAY

EVALUATION OF ITS CLINICAL VALUE[1]

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Donor-specific cytotoxic T cell activity was measured over a period of 5 years after transplantation using the cell-mediated lympholysis (CML) test in 124 recipients of unrelated kidney allografts who received conventional immunosuppressive therapy consisting of azathioprine and prednisone. Since patients with a functioning transplant frequently display donor-specific CML nonresponsiveness in vitro, we addressed the question of whether the CML status has a predictive value regarding the graft prognosis at any time interval until 5 years posttransplantation. From log-rank type analyses we conclude that the estimated relative risk calculated over the whole follow-up period of a CML-responder in the category of transplant rejectors is 1.25 with 95% confidence bounds between 0.94 and 1.65. Measurements of CML responder status during follow-up seem to have only limited prognostic value, although the relative risk is borderline significant when the analysis is restricted to the period between 2 weeks and 6 months posttransplantation.

The long-term immunologic acceptance of a histoincompatible organ graft, which is often assumed to reflect a tolerant state, could be measured in vitro by the use of the cell-mediated lympholysis (CML)* assay Peripheral blood lymphocytes from renal transplant patients with a well-functioning graft have often been shown to exhibit donor-specific CML-nonresponsiveness (CML-NR) while the CML reactivity against randomly chosen stimulator cells usually remains intact (*1–7*)

The exact mechanism(s) that underlie the development of such donor specific CML-NR after transplantation are not known, but evidence has been presented that the donor HLA system may regulate this phenomenon (*8*) Mechanisms that may be involved include the involvement of suppressor cells (*9–14*), antiidiotypic antibodies (*15, 16*), Fc receptor blocking antibodies (*17*), antiidiotypic T cells (*18*), veto cells (*19*), or clonal deletion (*13, 20, 21*) Since the observations of CML-NR are essentially retrospective evaluations in selective groups of patients, we decided to investigate the predictive value of the CML status for graft survival Here we present the results of 372 donor-specific CML studies in 124 renal transplant recipients in relation to allograft survival

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* Abbreviations CML, cell mediated lympholysis, CML NR, cell mediated lympholysis nonresponsiveness, CML R, cell mediated lympholysis responsiveness

MATERIALS AND METHODS

Patients PBLs from 124 renal patients who had received a first cadaveric renal transplant through the services of Eurotransplant were investigated for their cytolytic potential against donor splenocytes as well as against control cells From 69 patients additional pretransplant PBLs were available for study All patients had received one or more blood transfusion(s) before transplantation Immunosuppression consisted of prednisone and azathioprine Transplantation was considered successful if the recipient remained alive without (re)institution of dialysis Patients who rejected their grafts were studied till graft nephrectomy

In vitro studies Serial samples of recipients' PBLs were collected at several intervals up to 5 years after transplantation The time intervals of blood sample collection and the total number of samples (average 4, maximum 13) available for study varied for each patient The PBLs (i e 10^6 responder cells) were sensitized in vitro for 6 days against 10^6 irradiated splenocytes from the specific kidney donor as well as against 10^6 control cells from healthy unrelated individuals Depending on the amount of lymphocytes available, which was limited in most of the cases, either tissue culture flasks or 2 ml cluster wells were used, the ratio responder/stimulator cell however is identical in both culture conditions After the culture period, the effector cells were harvested and tested in the standard CML assay against their specific stimulator cells (i e splenocytes of the specific kidney donor and control cells of healthy unrelated individuals) as target cells

Donor lymphocytes were obtained from the spleen All patients' blood samples, the donor spleen cells, and the control cells were frozen and stored in liquid nitrogen until used

CML NR or CML R These terms are used to describe the CML nonresponsiveness or CML responsiveness, respectively, exhibited by the recipients' PBLs against the specific kidney donor splenocytes Almost all the recipients showed a normal cytolytic response to HLA incompatible control cells The few cases in which the response to the control cells remained low in repeated experiments were excluded from the analyses

The CML assay has been described in detail (*22*) The percentages of lysis were determined using phytohemagglutinin stimulated blast cells in a 4 hr ^{51}Cr assay Cytotoxicity (i e the amount of isotope released from ^{51}Cr labeled target cells) was determined and calculated according to the described method (*22*) Standard errors of the mean of triplicate determinations were less than 5% Positive and negative

assignments were made on the basis of a 10% specific ^{51}Cr release value and on a positive slope, i e the various effector to target cell ratios are plotted and must give an S shaped curve (or in the case of transforming the percentage of lysis to a log scale a straight line) All experiments were repeated at least twice at different effector to target cell ratios

Statistical analyses In order to assess the association between CML responder status during follow up and subsequent graft failure, a log rank type analysis was performed following the method outlined by Mantel and Byar (*23*) At each time point t (days posttransplantation) on which one or more failures occurred, the group of patients at risk was divided into two groups, CML NR or CML R, according to their last CML test done before time t (Patients without CML test before time t were considered to be not at risk) Next, for each group the expected numbers of failures (under the hypothesis that the probability of failure does not depend on the CML status) were computed in the usual way Then expected and observed numbers of failures were compared analogously to the procedure of the standard log rank test

Two analyses were carried out In the first one the endpoint was graft failures from all causes including death of the patient In the second analysis the endpoint was acute rejection Observations of graft failures with another cause and observations of patients who died with a functioning graft were treated as censored in this analysis

RESULTS

Longitudinal CML studies up to 5 years after transplantation were performed with the lymphocytes from 124 patients who had received a kidney transplant from an unrelated donor Three examples of serial investigations are shown in Figure 1 The period in which the CML responder status may convert from positive to negative differs between individuals, as exemplified in Figure 1

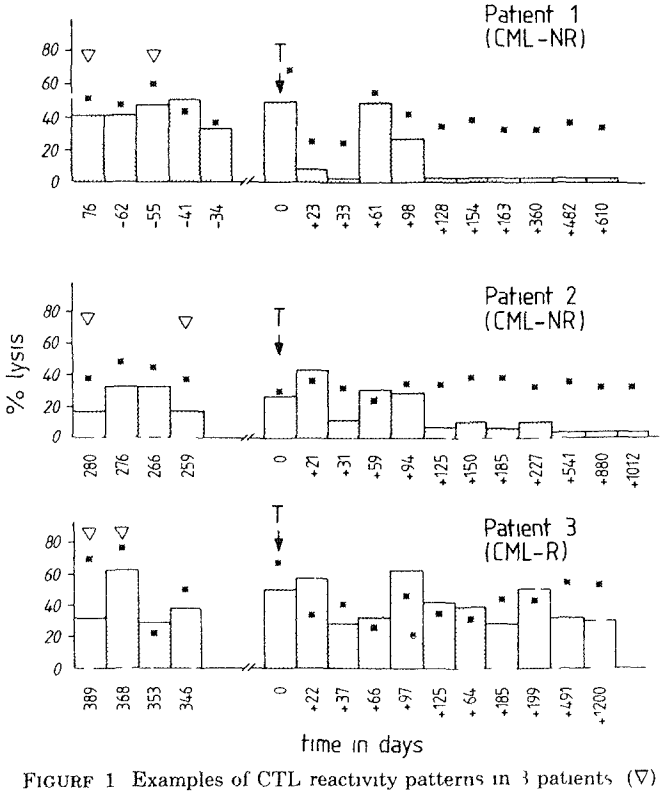

FIGURE 1 Examples of CTL reactivity patterns in 3 patients (▽) blood transfusion and (T) day of renal transplantation Bars indicate the percentage of lysis against the specific kidney donor splenocytes (*) percentage of anticontrol cell lysis (CML NR) CML Non Responder (CML R) CML Responder

TABLE 1 Observed and expected numbers of graft failures according to CML status and time posttransplantation

Time (days posttransplant)	CMI status NR[b]			CML status R[b]		
	N at risk	N obs failures	N exp failures	N at risk	N obs failures	N exp failures
2	19	0	0 24	60	1	0 76
6	24	0	0 30	55	1	0 70
7	25	0	0 32	53	1	0 68
12	30	1	0 37	52	0	0 63
13	31	1	0 38	50	0	0 62
14	31	0	0 39	49	1	0 61
15	31	0	0 39	48	1	0 61
19	32	0	0 41	46	1	0 59
20	31	0	0 40	46	1	0 60
21	32	0	0 42	44	1	0 58
34	33	0	0 44	42	1	0 56
36	33	1	0 45	41	0	0 55
37	32	0	0 44	41	1	0 56
40	32	0	0 44	40	1	0 56
42	32	0	0 45	39	1	0 55
80	30	0	0 43	40	1	0 57
89	30	0	0 43	39	1	0 57
95	30	1	0 45	37	0	0 55
104	30	1	0 45	36	0	0 55
110	29	0	0 45	36	1	0 55
133	35	1	0 52	32	0	0 48
153	35	0	0 53	31	1	0 47
177	36	0	0 55	30	1	0 45
376	49	0	0 66	25	1	0 34
438	53	1	0 71	22	0	0 29
562	59	1	0 76	19	0	0 24
627	60	1	0 75	20	0	0 25
739	67	0	0 78	19	1	0 22
748	67	0	0 79	18	1	0 21
810	69	1	0 79	18	0	0 21
830	69	1	0 79	18	0	0 21
871	69	1	0 78	19	0	0 22
912	68	1	0 78	19	0	0 22
1214	68	1	0 78	19	0	0 22
1243	64	1	0 78	18	0	0 22
1758	47	1	0 47	19	0	0 29
2007	44	0	0 71	18	1	0 29
2073	44	0	0 72	17	1	0 28
2205	43	1	0 74	15	0	0 26
2241	42	1	0 74	15	0	0 26
2421	40	1	0 74	14	0	0 26
Total[a]		19	22 92		23	17 84

[a] Log rank analysis calculated over the whole follow up period yield $P = 0\ 13$

[b] CML status according to the last previous measurement

In order to assess the relationship between CML status during follow up and the risk of graft failure, the log rank analysis was carried out Table 1 shows the observed and expected numbers of failures according to CML status at each time that a failure occurred Although a trend is visible (on average more rejections observed than expected in CML R patients), we could not accept the hypothesis that CML R patients have more graft failures than CML NR ($P = 0\ 13$) The Mantel Haenszel estimate of the relative risk (CML R versus CML-NR) was 1 25 with 95% confidence interval of 0 94–1 65 Since most renal transplants are lost because of acute rejection in the first 6 months after transplantation (*24*),

we carried out the same analysis for this time traject The results shown in Table 2 demonstrate that the number of graft rejections in the CML R group is higher than expected on the basis of random distribution of rejection cases between CML-NR and CML-R patients The effect is most prominent in the period between 2 weeks to 6 months posttransplantation, which just reached statistical significance ($P = 0\ 052$)

The statistical analysis as shown above was done based on the information that the graft was either "functioning" or "nonfunctioning " Therefore "nonfunctioning" grafts included acute rejections, which were about two thirds of all failures, recurrent original disease, chronic rejection, death of the patient, or other not further-identified causes of failures Consequently, the same statistical analysis was carried out on functioning renal allografts versus acute rejections only This analysis as shown in Table 3 demonstrates that although a trend is observed similar to the results of the analysis comprising all "nonfunctioning" grafts (Table 2), the data fail to reach statistical significance ($P = 0\ 085$, posttransplant period 15–153 days)

We also investigated whether the *pre*transplant CML status has predictive capability for the likelihood of posttransplant development of CML-NR The availability of *pre*transplant blood samples of 69 recipients provided the information concerning the donor-directed CTL reactivity *prior* to grafting Fifty one recipients showed CML reactivity against the splenocytes of the kidney donor Twenty eight of 51 patients who exhibited pretransplant CML reactivity developed donor-specific CML nonreactivity at different times posttransplant, whereas 23 patients persistently demonstrated donor-directed CTL activity, 15 pretransplant CML NR patients remained CML NR posttransplantation Three patients with acute irreversible rejection showed "reversed" CML conversion absence of pretransplant donor directed CTL activity but high levels of cytotoxic activity shortly after grafting prior to graft nephrectomy Acute irreversible graft rejection was observed within 7, 12, and 15 days, respectively, this "reversed conversion" of CML NR pretransplant to CML-R posttransplant might appear to be an indication for poor graft survival

TABLE 2 Results of log rank analyses for the relationship between CML status and graft survival[a] in some different time trajects

Period in days	CML NR[b] N obs	CML NR[b] N exp	CML R N obs	CML R N exp	*P* value	Estimated relative risk (95% confidence bounds)
0–2421	19	22 92	23	17 84	0 13	1 25 (0 94–1 65)
0 183	6	10 08	18	13 91	0 09	1 69 (0 92–3 09)
15–183	4	8 08	14	9 92	0 05	2 04 (0 99–4 17)
34 183	4	6 46	10	7 54	0 19	1 62 (0 79–3 33)
365–2421	13	13 5	5	4 48	0 78	1 04 (0 79–1 37)

[a] Endpoint is graft failure from *all causes* (death of a patient always being considered as graft failure)

[b] See legends Table 1

Whole period

TABLE 3 Results of log rank analyses for the relationship between CML status and graft survival[a] in some different time trajects

Period in days	CML NR[b] N obs	CML NR[b] N exp	CML R N obs	CML R N exp	*P* value	Estimated relative risk (95% confidence bounds)
0–2073	12	15 40	17	13 60	0 18	1 29 (0 89–1 86)
0–183	5	7 95	14	11 05	0 17	1 60 (0 82–3 11)
15–183	3	6 19	11	7 81	0 085	2 08 (0 90–4 78)
31 183	3	4 57	7	5 43	0 32	1 53 (0 66–3 53)
365–2073	7	7 45	3	2 55	0 75	1 06 (0 73–1 55)

Endpoint is *acute rejection* (observations of patients who die with functioning grafts are censored)

See legends Table 1

Whole period

DISCUSSION

In vitro donor specific cell-mediated lympholysis nonresponsiveness exhibited by cells from recipients with well-functioning kidney allografts is a generally observed association (*1–7*). All studies except one (*6*) were carried out with a short-time follow-up Notwithstanding these observations, we assessed to what extent this apparent in vitro reflection of acquired tolerance can be explained by selection for patients who get the chance to develop CML-NR The availability of over 350 CML studies in 124 renal transplant patients with a follow-up period of approximately 5 years enabled us to investigate whether the CML NR predicts graft survival during follow-up

By assessing the correlation of the outcome of the CML tests and graft survival for different time intervals, it appeared that only the posttransplant period between 2 weeks and 6 months showed a (marginally) significant correlation ($P = 0\ 05$) between CML-NR and graft survival on one hand, and CML-R and graft lost on the other hand; the data failed however to reach statistical significance when analyzing only the acute rejections ($P = 0\ 085$, Table 3) Nevertheless, these trends could be of interest in view of the clinical observation that most acute rejection episodes occur within this time period (*24*) The results obtained with the 69 patients serially monitored from *pre-* to posttransplantation demonstrated that pretransplant donor-directed CTL activity is not a contraindication for transplantation and does not preclude the development of CML NR posttransplantation Conversely, pretransplant CML-NR converting shortly after transplant to CML-R was in 3 out of 3 cases associated with acute rejection and graft loss

It also appeared from our studies that several patients had a well-functioning graft while demonstrating in vitro donor-specific CML activity, likewise a number of patients rejected their graft despite their CML-NR status The situation in which patients with a well-functioning graft show in vitro donor-specific CML activity is compatible with the experimental animal studies in which specific cytotoxic T cells were found in nonrejected rat kidneys (*25*) The cellular mechanism of graft destruction is probably composed of phenotypically and functionally different T cell subsets directed against MHC class I and class II transplantation antigens, as was recently reported by Miceli et al (*26*) and by Bonneville et al (*27*) These observations are consistent with the findings that cytotoxic T effector cells are not the sole mediators of graft rejection as was first observed by Loveland et al (*28*) Although these and numerous experimental animal studies that followed (*29*), which were designed to unravel the effector mechanisms of allograft rejection, concern skin grafting in nonimmune suppressed animals, they provide important insights into the complexity of immunologic responses in human allografting. Effector mechanisms operating in vascularized human allograft rejection are also dependent on the genetic constitution of the recipient (*30*), and antigen expression status of the graft (*31*,

32). Furthermore, pretransplant blood transfusions are known to have a beneficial effect on kidney graft prognosis (*33, 34*) We reported earlier that CML-NR occurs with the same frequency in both single and multitransfused patients (*4*). Recently, we designed a prospective study to analyze blood-transfusion-induced changes in cellular and humoral immunity (*35*) Preliminary results of this ongoing study show that if the transfusion donor and recipient are mismatched for both HLA-DR antigens, the recipient is immunized resulting in a transient increase of cytotoxic activity, after an HLA DR shared transfusion, the in vitro test remained unchanged or slightly decreased. However in none of cases studied so far, has it led to CML-NR

A method of monitoring the graft certainly is to perform graft biopsies (*36*) or fine-needle aspirates (*37*) The in vitro monitoring by measurement of the donor-directed cytotoxic T cell activity as described herein may provide further information regarding acute rejection Although predictions regarding the likelihood of the occurrence of posttransplant CML-NR must be made with great caution, posttransplant monitoring using the CML assay may at least for the first 6 months after transplantation be helpful for the interpretation of clinical events and possibly for the design of therapeutic strategies

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MECHANISMS OF INSULIN RESISTANCE AFTER KIDNEY TRANSPLANTATION[1]

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In order to study the effect of corticosteroids on energy metabolism in immunosuppressed patients after kidney transplantation, we have examined glucose utilization, energy expenditure, and lean body mass in 10 kidney-transplanted patients receiving steroids (methylprednisolone 8.2±1.5 mg/day) and in 10 healthy age- and weight-matched control subjects. Glucose utilization was measured during euglycemic insulin clamp in combination with indirect calorimetry and infusion of [^{3}H-3]-glucose, while β-cell function was measured during a hyperglycemic clamp. The kidney-transplanted patients were resistant to the glucoregulatory effect of insulin, as demonstrated by a 25% reduction in total glucose disposal compared to control subjects. This defect was almost completely accounted for by a defect in storage of glucose as glycogen (3.3±0.5 vs. 5.0±0.5 mg/kgLBM·min; $P<0.05$). The reduction in nonoxidative glucose disposal was associated with reduced lean body mass and incapacity to release energy as heat after infusion of insulin, i.e. thermogenic defect. In contrast, oxidation of glucose and lipids was not influenced by steroid therapy. Futhermore, suppression of hepatic glucose production was normal, and insulin secretion was normally enhanced in relation to the degree of insulin resistance in the steroid-treated patients. In conclusion, steroid-induced insulin resistance in kidney-transplanted patients is due to alterations in the nonoxidative pathway of glucose metabolism. These findings raise the question of whether steroid therapy directly influences glycogen synthase in man.

Despite the introduction of cyclosporine and other new drugs, corticosteroids have been the cornerstone in immunosuppressive therapy after kidney transplantation. Steroids have been known to induce insulin resistance in experimental animals (*1, 2*) and in man (*3–5*). Several mechanisms have been suggested to explain the steroid-induced insulin resistance, e.g. decreased insulin receptor number and affinity (*6, 7*), impaired peripheral glucose uptake (primarily in muscle) (*8*), impaired suppression of endogenous glucose production (*9*), and activation of the glucose/fatty acid (FFA)* cycle initially proposed by Randle et al. (*10*). The glucose/FFA cycle is based upon the concept of substrate competition, i.e. increased utilization of FFA leads to decreased utilization of glucose and vice versa. In addition to effects on glucose metabolism, steroids are known to induce protein catabolism (*11*). The clinical consequence is wasting of muscle tissue. The majority of glucose after a meal is taken up by muscle tissue (*12, 13*), where it is metabolized by either of two pathways: oxidation to carbon dioxide and water, or storage as glycogen. Total body glucose metabolism has been shown to correlate with the muscle mass (*14*), but it is not known whether insulin resistance correlates with muscle wasting during chronic steroid therapy.

The clinical endpoint of insulin resistance is diabetes mellitus. The incidence of secondary diabetes after kidney transplantation has markedly increased over the past years (*15, 16*). There may be several explanations for this phenomenon: inclusion of older patients in transplantation programs and the use

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* Abbreviations FFA, glucose/fatty acid, HGP, hepatic glucose production