Cytokine analysis of human renal allograft aspiration biopsy cultures supernatants predicts acute rejection

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Abstract

Background. A Th1 response is said to be associated with transplant rejection and Th2 with tolerance, although this is not agreed by all. Cytokines evaluation in peripheral blood and urine in kidney transplants produces variable results. We hypothesized that measurement of major cytokines involved in Th1/Th2 paradigm on transplant renal-infiltrating cells could bring valuable scientific and clinical information.

Methods. Fifty-six adult cadaver kidney transplants were subdivided into 21 stable patients (group A), 22 suffering acute rejection (group B), 10 with chronic rejection (group C) and three with CMV disease (group D). Fine-needle aspiration biopsies were cultured and their supernatants analysed for IL-2, IL-4, IL-10 and IFN-γ.

Results. Group A produced small amounts of both IL-2 and IL-10 while group B synthesized significantly higher IL-2 and significantly lower IL-10 amounts than group A. Group B produced significantly more IL-2 than A on day 7 post-transplantation, several days before rejection supervened. Group C produced IL-10 and very low amount of IL-2. Group D produced both IL-2 and IL-10. We did not find any IL-4, and IFN-γ was present in a few samples. For IL-2, sensitivity, specificity, negative and positive predictive values for acute rejection were 100, 87.2, 94.7 and 83.3%, respectively.

Conclusions. Cytokine analysis in fine-needle aspiration biopsy cultures supernatants is a very useful immunological screening method for kidney transplants. IL-2 synthesis on day 7 post-transplantation reliably predicted the risk of impending acute rejection during the first weeks. The cytokine pattern suggests that acute rejection is associated with Th1, stable patients with Th0/Th2, and chronic rejection with Th2 patterns.

Key words: FNAB; interleukins; lymphocytes; rejection; T helper; tolerance; transplantation

Introduction

Activated CD4⁺ T cells play a pivotal role in the induction of allograft rejection [1]. They have been subclassified on the basis of their phenotypic pattern of cytokine production: Th0 cells (IL-2, IL-4, IFN-γ, lymphotoxin), Th1 (IL-2, IFN-γ, lymphotoxin) and Th2 (IL-4, IL-5, IL-6, IL-10). Th0 is the common precursor to both Th1 and Th2 [2,3]. The cytokine milieu at the time of antigenic stimulation can greatly influence the development of Th1 and Th2 subsets as there is no Th0 commitment to either subset [3]. Th1 maturation requires IL-12/IFN-γ while Th2 needs IL-4 [4]. Several groups have presented data linking a Th1 cytokine pattern to allograft rejection and Th2 cytokine to transplant tolerance [5–12]. However, others have reported conflicting results [13–15], which suggests that the Th1/Th2 paradigm may not be sufficient to fully explain the mechanisms underlying allograft rejection and tolerance.

The measurements of cytokines from renal transplant patients either in peripheral blood or in urine have produced variable results, with some pointing to their potential use in the diagnosis of renal graft rejection [16,17], and others failing to reproduce these findings [18,19].

Fine-needle aspiration biopsy (FNAB) is a reliable and safe diagnostic tool for the immunologic monitoring of kidney transplants [20–22]. Previously we have shown that FNAB cell cultures have significant different proliferative abilities whether they come from stable kidney transplant patients or from acute rejecting patients [23]. We hypothesized that these different proliferative responses could reflect discriminant cytokines patterns of production, following our previous report on chemokines synthesis by FNAB cell cultures [24], which could help both the diagnosis of different pathologic conditions.
post-kidney transplantation and mark an evolution to allograft tolerance. This is the report of our studies.

**Subjects and methods**

Fifty-six adult cadaver renal transplants recipients, 35 male and 21 female with an age range of 16–52 years entered the study. All were receiving renal replacement therapy before transplantation, 53 on haemodialysis and three on CAPD. The original renal diseases consisted of chronic glomerulonephritis/unknown (19 cases), hereditary nephritis/tubulointerstitial nephritis (17), IgA nephropathy (5), polycystic renal disease (5), diabetic nephropathy (5), rapidly progressive glomerulonephritis (2), systemic lupus (1), membranoproliferative glomerulonephritis (1), Schönlein–Henoch purpura (1). All were first kidney transplants with the exception of three recipients of a second graft. The transplant recipients and the donors were typed by microlymphotoxicity tests using well standardized alloantisera.

**Immunosuppression**

Fifty-three patients were treated with triple therapy (CsA–Aza–Pred) and the three second transplants received quadruple therapy, which included rabbit/horse ATG for the first 7 days post-surgery. The b.i.d. oral CsA dose was adjusted according to CsA trough levels measured by monoclonal TDX® assay from Abbott. Acute rejection episodes were treated by 3–4 i.v. 500-mg methylprednisolone pulses, and by ATG/OKT3 for 7–10 days in steroid-resistant cases and whenever the biopsies were classified grade IIb or greater following Banff criteria.

- **group A**, 21 patients rejection-free during their first year
  - **group B**, 22 patients with acute rejection episodes
  - **group C**, 10 patients with chronic rejection
  - **group D**, 3 patients with cytomegalovirus (CMV) disease.

Acute rejection episodes were confirmed by core renal biopsy in all but one case where diagnosis was based on FNAB findings. Also the rejection diagnosis was confirmed by a positive response to antirejection treatment, with the exception of six irreversible acute rejection cases. Chronic rejection was diagnosed in cases of steady and slow deterioration of transplant function after exclusion of other causes of graft deterioration and confirmed by core renal biopsy findings in six cases. CMV disease was diagnosed when classical clinical symptoms and signs appeared with positive cytomegalovirus early antigenemia.

FNAB were done according to the methods described by Haýry [20] on days 7 and 30 post-transplantation in stable cases, whenever a rejection episode was suspected, during stable periods of chronic rejection, and on the first day of admission in CMV disease. The FNAB were done 2 h following morning dose of the immunosuppressive drugs. The samples were aspirated into 6 ml of RPMI medium with hparin lithium at 125 U/ml. After red blood cell lysis the crude cell suspensions were adjusted to a final concentration of $5 \times 10^7$ cells/200 ml. The cultures were done in RPMI medium supplemented with penicillin, gentamicin and 1-glutamine, 10% autologous serum (derived from blood drawn just before morning dose of the drugs) and 10 U/ml of rIL-2. The incubation was done at 37°C and 5% of CO₂ during 48 h. Then the cultures supernatants were collected and kept at −70°C until tested.

The supernatants were assayed for IL-2, IL-4, IL-10 and IFN-γ production using ELISA kits (Endogen®, Cambridge, MA) according to manufacturer’s instructions. Also, serum used as culture medium supplements were assayed for IL-2 and IL-10. The ELISA kits sensitivities for IL-2, IL-4, IL-10, and IFN-γ were <6 pg/ml, <2 pg/ml, <3 pg/ml, and <2 pg/ml respectively.

The results were analysed by an unpaired Student t test (serum creatinine and blood CsA), Friedman ANOVA for paired samples and Kruskal–Wallis ANOVA for unpaired samples (Statistica® from Statsoft). This study was approved by the local Ethics Committee, and informed consent was obtained in all cases.

**Results**

We examined IL-2 production in 21 samples on day 7 FNAB culture supernatants and 18 samples for day 30 post-transplantation in group A. The respective medians $\pm$ SEM were 15 $\pm$ 175 pg/ml and 13.5 $\pm$ 176 pg/ml. The high SEM were explained by three high producers on day 7 cultures and two (one of the three day 7) on day 30 cultures; their IL-2 values were 252, 322, 1600, and 330, 1455 pg/ml respectively. These four patients had good renal function at both periods, with CsA blood levels within our therapeutic range (creatines/CsA: 1.0/250, 3.1/206, 2.0/106, 1.7/220, 1.8/261 mg/dl and ng/ml respectively). The four have remained rejection-free 1 year post-transplantation, although one of them suffered several spontaneously reversible graft dysfunction episodes as well as urinary-tract infections.

For group B, we did 16 tests with day 7 FNAB culture supernatants and 22 tests with FNABs done at the beginning of their rejection crisis. The 22 acute rejection episodes occurred during the first 60 days post-transplantation with the exception of one diabetic case which happened 510 days post-transplantation, 90 days following steroids withdrawal. The day 7 FNABs were done 10 $\pm$ 9 days before rejection. IL-2 production at day 7 was 438 $\pm$ 387 pg/ml; the values measured in culture supernatants of FNAB done at the beginning of rejections were 871 $\pm$ 387 pg/ml. Six of the 22 suffered irreversible rejections, the corresponding IL-2 were 1140 $\pm$ 318 pg/ml; in supernatants from the other 16 who suffered reversible graft rejections we measured 441 $\pm$ 214 pg/ml.

We performed 10 tests in group C. FNABs were done 615 $\pm$ 185 days post-Tx. Eight of the 10 patients lost their graft function during the year following FNAB. IL-2 was 2.5 $\pm$ 92 pg/ml. Two showed 370 and 215 pg/ml, quite interestingly both still retain their kidneys with plasma creatinine oscillating around 3.0 mg/dl. Unfortunately we do not have day 7 FNAB from any of these patients.

Three patients from group D suffered CMV disease during the first 60 days post-transplantation. IL-2 were...
844, 485 and 1446 pg/ml; they fully recovered after a therapeutic course of gancyclovir.

The four groups were similarly analysed for IL-10 production. From group A, day 7 FNAB supernatants, we measured 22 ± 32 pg/ml and day 30 supernatants 5 ± 29 pg/ml. For group B, day 7 supernatants contained 2 ± 10 and at the start of rejection 2 ± 12 pg/ml. For the six patients suffering from irreversible rejection, IL-10 was 1 ± 10.9 pg/ml. For group C, IL-10 production was 32 ± 37 pg/ml. The three with CMV disease showed 112, 115 and 160 pg/ml of IL-10.

We further analysed the results from group A and B with exclusion of groups C and D. IL-2 ≥ 250 pg/ml was assumed as cut-off value for acute rejection diagnosis. The sensitivity was 22/22, 100%, specificity was 34/34 ± 5, 87.2%. The positive predictive value was 22/22 ± 5, 81.5%, and negative predictive value was 34/34, 100%. Considering day 7 samples from group A and B, and applying the same cut-off value for IL-2, the positive predictive value for subsequent rejection was 15/15 ± 3, 83.3% and the negative predictive value was 18/18 ± 1, 94.7%.

We tested the correlations between IL-2 vs blood CsA. Group A, day 7 and day 30 they were −0.06 and 0.37. For group B, day 7 and rejection day, they were 0.42, P = 0.028 (Figure 1), and 0.19 respectively. For IL-10 they were 0.04, −0.31, 0.10 and −0.04, groups A and B respectively. For group C the correlations between IL-2, IL-10 vs CsA were −0.1 and −0.22 respectively. In addition there was no significant correlation between serum creatinine versus either IL-2 or IL-10 for group A, B, and C. For group B we found a positive correlation between day 7 vs rejection day for IL-2, 0.53, P = 0.031 (Figure 2). In Table 1 we present plasma creatinine and blood CsA levels of the four groups. In Table 2 the analysis of the results by ANOVA are shown.

FNAB culture supernatants from groups A, B, C and D did not show any trace of IL-4.

IFN-γ was only found in two patients from group A, one of the two producing IFN-γ both from day 7 FNAB and day 30 FNAB. Five culture supernatants from group B contained small amounts of IFN-γ (3, 13, 22, 45, 55 pg/ml). This small number of positive samples for IFN-γ prevented any meaningful statistical analysis. Groups C and D were not studied for IFN-γ.

Serum used as culture medium supplements were analysed for IL-2 and IL-10. We did not find IL-2 in any serum sample. Concerning IL-10, group A day 7 and day 30, median ± SEM were 6.0 ± 6.3 pg/ml and 6.5 ± 14.1 pg/ml respectively; in group B day 7 and rejection day we measured 8.0 ± 18.4 pg/ml and 7.8 ± 14.7 pg/ml, in group C, 5.0 ± 6.4 pg/ml and the three patients from group D, 65, 82 and 77 pg/ml.

Discussion

Several conclusions can be drawn from our results. First, our FNAB cultures have the ability to produce several cytokines, contrary the findings by Yard et al. [25] who showed that unstimulated graft-infiltrating cells cultures were unable to synthesize any cytokine. We supplemented our cultures with 10 U/ml of rIL-2 only, and this amount is less than half the dose used for propagation of activated T cells from allograft biopsies [26]. We decided to use autologous serum as culture medium supplement as this might reflect the in vivo milieu more closely while at the same time not eliminating the possible interference of CsA levels. However, as can be seen by correlation analysis between supernatant IL-2 and CsA, cyclosporin blood values did not have a major influence in stable patients FNAB culture IL-2, and quite unexpectedly we observed a positive correlation within rejection group. The absence of IL-2 and the amount of IL-10 measured

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**Table 1. Serum creatinines and trough blood CsA levels in group A (stable patients), group B (acute rejection patients), group C (chronic rejection), group D (CMV disease)**

<table>
<thead>
<tr>
<th></th>
<th>Creatinine (mg/dl)</th>
<th>CsA (μg/ml)</th>
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<tbody>
<tr>
<td>7 d, group A</td>
<td>2.9 ± 3.3</td>
<td>210 ± 220</td>
</tr>
<tr>
<td>30 d, group A</td>
<td>1.7 ± 3.2</td>
<td>220 ± 56</td>
</tr>
<tr>
<td>7 d, group B</td>
<td>4.2 ± 3.1</td>
<td>182 ± 85</td>
</tr>
<tr>
<td>group B</td>
<td>4.3 ± 2.5</td>
<td>169 ± 49</td>
</tr>
<tr>
<td>group C</td>
<td>4.2 ± 1.2</td>
<td>180 ± 99</td>
</tr>
<tr>
<td>group D</td>
<td>1.7, 1.8, 3.2</td>
<td>255, 279, 215</td>
</tr>
</tbody>
</table>

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![Fig. 1. Correlation of CsA vs IL-2, Gr. B, day 7 post-transplantation, r = 0.42.](image1)

![Fig. 2. Correlation between day 7 and rejection day for IL-2, r = 0.53, P = 0.031.](image2)
in serum samples could not explain the different cytokine production patterns by FNAB cultures. Also, different to conditions of Yard et al. [25], our system is a coculture, the main cells involved are donor parenchymal renal cells with host mononuclear cells and this may account for the differences in our results. Actually one might consider that we are performing a mixed lymphocyte culture variant, but in our test donor alloantigen is provided by either renal cells or more probably, by recipient antigen-presenting cells infiltrating the kidney graft. Alternatively, procedures for graft-infiltrating lymphocytes isolation from core renal biopsies could interfere with cells capacity to synthesize cytokines. Some doubts could be raised by a possible FNAB sample contamination by blood. However, we looked at the samples under light-microscopy following a modified Romanowski stain, and only those samples that did not show significant blood contamination were subjected to culture (this was assessed according Hary classification [20]). Furthermore we have run a study by flow cytometry of the FNAB samples together with the corresponding peripheral blood and this has demonstrated the absence of significant blood contamination, as the analysed lymphocyte subsets presented several differences when we compared FNAB to peripheral blood lymphocytes [27].

IL-2 and IL-10 analysis of FNAB cultures were very rewarding as a distinct pattern emerged from stable and rejecting patients. Group A (stable), showed a Th0/Th2 cytokine pattern, with a few exceptions (3/21) from day 7 samples. This group has enjoyed a rejection-free first year post-transplantation, including the three high-IL-2 producers. We suspect that these three patients are cases of intragraft activation without clinical consequences [28], a poorly understood phenomenon that has been previously reported. The blood CsA levels measured on these three patients were within the therapeutic range.

Group B displayed a predominant Th1 cytokine pattern. IL-2 values ranged from 370 pg/ml up to 1640 pg/ml. Interestingly, irreversible acute rejection episodes were accompanied by significantly higher IL-2 values than in reversible acute rejection episodes. The almost complete IL-10 inhibition in rejection cases compared to values measured in stable cases points to its important role in human renal transplantation and goes against a predominant part for IL-10 in severe graft rejection, as others have suggested [29]. However, we cannot exclude that IL-10 might be the predominant cytokine in ‘pure humoral’ rejection as we have only one such case studied. This patient, a 17-year-old male lost his graft following a Banff grade III rejection, presenting disseminated arteries thrombosis with mild to moderate interstitial mononuclear infiltration, and his IL-2/IL-10 values at start of rejection were 1018/41.2 pg/ml respectively. Actually this IL-10 value was the second highest measured among group B, and the highest was seen in a female recipient suffering a severe Banff grade IIb rejection successfully treated with a course of horse ATG. However, her IL-2 reached up to 993 pg/ml while her IL-10 was 89 pg/ml. We speculate that IL-10 might be synthesized by monocyte–macrophage population present in our coculture. Interestingly, there is a significantly increased presence of CD14+ cells in our FNAB taken from steroid-resistant acute rejection cases [24].

FNAB done on day 7 post-transplantation in group B, 10±9 days before the start of the rejection crisis, showed a significantly higher IL-2 production than their counterparts from group A. This important finding suggests that immunological activation is already on course at day 7 and ultimately would lead to acute rejection crisis. The correlation between day 7 IL-2 and rejection IL-2 was 0.59, P = 0.014 (Figure 2). CsA blood levels were not responsible for these results as there were no significantly differences comparing day 7 CsA levels from group A with day 7 in group B (Table 1). Only one of 16 patients deviated from this behaviour, the other 15 had IL-2 from 270 up to 1700 pg/ml. The outsider produced just a small amount of IL-10 with no IL-2 and he was the first patient to enter this study. We cannot exclude that a preservation problem affected his supernatant. The day 7 IL-2 synthesis may act as a warning of the risk of an impending rejection crisis, and at the same time potentially point to a CsA failure as an IL-2 synthesis inhibitor. One may then preempt this situation by increasing the CsA dose, specially in those with low to normal blood levels, or to add a new immunosuppressive drug.

Chronic rejection patients showed Th2 cytokine pattern. This is in agreement with a recent report of intragraft cytokine mRNA expression [30] where the authors did not observe IL-2 upregulation, but rather IL-10 from core renal biopsies. We are quite confident of the chronic rejection diagnosis of the four cases without renal biopsies. All of them lost their grafts. Their original renal diseases were vesicoureteral reflux [2], IgA nephritis and chronic glomerulonephritis. One

<table>
<thead>
<tr>
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<th>Day 7A vs day 7B</th>
<th>Day 30A vs B</th>
<th>B1 vs B2</th>
<th>B vs C</th>
<th>Day 7A vs day 30A</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>&lt;0.0001</td>
<td>0.0016</td>
<td>0.032</td>
<td>0.0001</td>
<td>0.31</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.012</td>
<td>0.37</td>
<td>0.73</td>
<td>0.005</td>
<td>0.034</td>
</tr>
<tr>
<td>CsA</td>
<td>0.53</td>
<td>0.16</td>
<td>0.065</td>
<td>0.26</td>
<td>0.46</td>
</tr>
</tbody>
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A, rejection-free; B, acute rejection; B1, reversible rejection; B2, irreversible rejection; C, chronic rejection.
could argue that IL-10 was produced by monocyte-macrophage but even so, one would expect that T lymphocytes exposed to IL-10 in the absence of IL-2 would deviate to a Th2 pattern, probably.

The similar IL-10 values measured in stable and chronic rejecting patients highlights the extreme complexity of this cytokine. IL-10 is produced by T and B lymphocytes and antigen-presenting cells [31] and is endowed with both stimulant and depressant effects on the immune cascade. IL-10 downregulates B7 [32] and MHC class II expression on macrophages [33], blocks IL-12 synthesis by macrophages [34], inhibits cell proliferation stimulated by macrophages and blocks IFN-γ release [35]. However, following repeated antigen stimulation there is a predominant IL-10 synthesis [36]. IL-10 stimulates proliferation and MHC II expression on B lymphocytes and promotes FcγRI expression on monocytes, and consequently antibody-dependent cell cytotoxicity [37], all probably causally related to chronic rejection process. We cannot explain the significantly higher IL-10 synthesis on day 7 vs day 30 in group A, but this might be the early sign of Th2 deviation or alternatively, monocytes-macrophages could be the producing cells, and by day 30 they are not seen anymore at significant numbers in our samples [24].

Three patients suffering from CMV disease presented high IL-2 values, as it would be expected in anti-viral response [38] and well above our cut-off value for acute rejection. We found very interesting the accompanying high values of IL-10 production, that we speculate could have played a part in preventing an ensuing acute rejection crisis.

IL-4 was not produced by FNAB cultures. IL-4 is said to be a strong marker of Th2 deviation and its ratio with IFN-γ has been pointed as the best mark of T lymphocyte subtype commitment [1]. However, in one animal model, neutralizing IL-4 through administration of a soluble IL-4 receptor leads to graft prolongation rather than hastened rejection [39]. CsA and FK506 have both the ability to suppress IL-4 production, specifically FK506 is a very strong inhibitor [40]. This could be an indirect signal that IL-4 does not play a dominant role in human transplantation.

IFN-γ was absent from most of FNAB cultures, only two of 22 of stable patients and five of 22 rejecting ones produced small amounts. This is agreement with Kirk et al. [41]. They found IFN-γ secretion by graft-infiltrating lymphocytes cells recovered from rejecting kidney transplants was not useful for diagnosis, as it was produced by a minority in reversible rejections and half of six irreversible cases. Our supernatants were collected at 48 h of incubation; we do not know if had we measured IFN-γ later we would find different results, as is suggested by Danzer et al. [42], who measured it at different times of MLR incubation and reported the highest values at 6 days of culture. On the other hand, our findings could reflect rapid reuptake of IFN-γ by cells in culture, explaining differences between mRNA and protein measurements.

In summary, renal transplant FNAB culture supernatants analysis showed a Th0/Th2 cytokine pattern in stable patients, predominant Th1 during acute rejection, Th2 during chronic rejection and Th0 at start of CMV disease not followed by acute rejection. More importantly, IL-2 produced by FNAB done on day 7 post-transplantation reliably predicted an ensuing rejection episode and IL-2 on rejection day signalled irreversible rejection. In case our results are confirmed in a larger group of patients, this test could prove a very useful method for immunological follow-up and an adjunct to guide immunosuppressive strategies in renal transplantation.

References


36. Croft M, Swain SL. Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. *J Immunol* 1995; 154: 4269–4282


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