Interactions between cyclosporine A and adenosine in kidney transplant recipients

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Interactions between cyclosporine A and adenosine in kidney transplant recipients. Adenosine is involved in a large number of physiological processes including immune response and vasomotor function. But its precise involvement in renal physiology is poorly understood. We have investigated the putative relationships between cyclosporine A (CsA) and adenosine (ADO) metabolism in kidney transplant recipients (KTR). We first compared ADO plasma levels in three groups of patients and in 10 controls: the first group (N = 14) was composed of CsA-treated KTR; the second group (N = 5) was KTR not treated with CsA, and the third (N = 6) was chronic kidney failure patients. We also measured ADO plasma level in two KTR treated with FK506, a CsA analog. ADO plasma levels in CsA-treated KTR were significantly higher (mean 0.76 \( \mu \)g/ml) than in the control group (mean 0.31 \( \mu \)g/ml; Mann-Whitney test, \( P < 0.001 \)). ADO plasma levels in KTR not treated with CsA were in the same range as those in controls. Finally, the ADO plasma level was increased in the two FK506-treated patients. We also investigated the action of CsA on ADO plasma degradation and uptake by erythrocytes in vitro. No interaction between adenosine deaminase and CsA was found because CsA, in the presence of adenosine deaminase, did not modify the plasma half-life of ADO. Conversely, in the presence of CsA (500 and 1000 ng/ml), the uptake of ADO by erythrocytes was significantly decreased in adenosine deaminase-free samples (analysis of variance, \( P = 1.8 \times 10^{-3} \) and 1.2 \( \times 10^{-4} \), respectively). We conclude that ADO plasma levels are significantly increased and correlate with CsA blood level in CsA-treated KTR, and that these high levels are due to CsA inhibition of ADO uptake by red cells. Since ADO and metabolites have well known immunosuppressive and vascular effects, ADO is likely to participate in the immune defect and in the vasoconstriction induced by CsA.

In 1976 Borel et al [1] discovered cyclosporine A (CsA), a new antilymphocytic agent, and in 1978 Calne et al [2] demonstrated that CsA had marked beneficial effects in cases of organ transplantation, which have been interpreted in terms of immuno-presssive effects [1–4]. However, the precise molecular mechanism of CsA immunosuppression is unclear.

Adenosine (ADO) may be implicated in CsA immunosuppressive effects, since ADO is a strong natural immunosuppressor [5, 6] and since adenosine deaminase inhibitors have beneficial effects in several experimental models of acute renal failure, both nephrotoxic and hemodynamic [20, 21]. ADO has strong protective effects against tissue ischemia [reviewed in 22], and it is well established that CsA induces renal vasoconstriction [8].

In light of these findings, the aim of the present study was twofold. First we measured ADO plasma levels both in kidney transplant recipients (KTR), receiving CsA therapy or not, and in patients with chronic kidney failure and compared them with those in control subjects; we also investigated ADO plasma level in two KTR under FK506, a CsA analog [4, 23, 24]. Second, we investigated the effects of CsA on the half-life of ADO in vitro to determine if CsA can modulate the action of adenosine deaminase, and/or if CsA can modify ADO uptake by red blood cells. These cells were chosen because their rapid up-take of ADO is the prime cause of the very low concentration of ADO in human blood [25]. Indeed, degradation to inosine by adenosine deaminase and RBC uptake are the two principal means of eliminating ADO in whole blood [25, 26].

METHODS

Patients were recruited in the Nephrology Department (Sainte Marguerite Hospital, Marseille, France). Twenty-one KTR (14 CsA-treated, 2 FK506-treated, 5 non-CsA non-FK506-treated), six patients with chronic renal failure and ten control subjects participated in the study (Table 1). Among the fourteen CsA treated patients, ten were under triple immunosuppressor therapy including prednisone and azathioprine (Table 1) and four received prednisone only. Ten of these fourteen were receiving...
antihypertensive drugs. The two FK506-treated patients were under prednisone and did not receive antihypertensive drugs. The five non-CsA-treated patients were under double immunosuppressive therapy (prednisone, azathioprine); two of them received antihypertensive drugs. Patients with chronic renal failure were not under immunosuppressor or antihypertensive drugs. Controls were healthy subjects without any medication. For both patients and controls, the ingestion of coffee or tea was suspended 72 hours before samples were taken. Patients treated with papaverine, indomethacin or dipyridamole were excluded from the study.

Adenosine assay

Adenosine (crystallized, 99% pure), adenosine deaminase (calf intestine, specific activity 200 IU/mg) and dipyridamole (5 mg/ml) were obtained from Boehringer Mannheim (France). Inosine (99% pure), α,β-methylene-adenosine-5′-diphosphate (AOPCP) and deoxycoformycin (Pentostatin) were from Lederle. 9-Erythro (2-hydroxy-3-nonyl) adenine (EHNA) was from Burroughs Welcome. Heparin (25 IU/ml) was from Choay Laboratory. Methanol and other reagents were obtained from Merck (France). The reversed phase chromatography column (Merck Lichrospher C18, 250 × 4 mm) was obtained from Merck (France).

Table 1. Principal biological parameters of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Kidney transplant recipients (CsA treated)</th>
<th>Kidney transplant recipients (non-CsA treated)</th>
<th>Kidney transplant recipients (FK506 treated)</th>
<th>Chronic kidney failure patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>46/14</td>
<td>39/14.8</td>
<td>51</td>
<td>51/12</td>
<td>36/11</td>
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<tr>
<td>Gender</td>
<td>4F/8M</td>
<td>4F/1M</td>
<td>1F</td>
<td>2F/4M</td>
<td>5F/5M</td>
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<tr>
<td>Duration of the graft months</td>
<td>32/30</td>
<td>158/117</td>
<td>2</td>
<td></td>
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<tr>
<td>Serum creatinine μM</td>
<td>142/42</td>
<td>220/133</td>
<td>168</td>
<td>146/9</td>
<td>80/21</td>
</tr>
<tr>
<td>BUN mM</td>
<td>9.3/2.8</td>
<td>15.7/9.8</td>
<td>10.8</td>
<td>10.2/1.7</td>
<td>5.3/2.5</td>
</tr>
<tr>
<td>12 Hour whole blood CsA or FK506 trough levels ng/ml</td>
<td>227/88</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Hour whole blood CsA peak levels ng/ml</td>
<td>831/110</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ADO level μM</td>
<td>0.72/0.22</td>
<td>0.26/0.14</td>
<td>1.3</td>
<td>0.37/0.14</td>
<td>0.31/0.13</td>
</tr>
<tr>
<td>Mean blood pressure mm Hg</td>
<td>100.3/10.08</td>
<td>106/13.2</td>
<td>96</td>
<td>94/8</td>
<td>80.7/10.09</td>
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<tr>
<td>Prednisone mg/day</td>
<td>12.1/2.6</td>
<td>18/2.7</td>
<td>15</td>
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<tr>
<td>Azathioprine mg/day</td>
<td>64.3/31</td>
<td>65/28</td>
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</table>

Data are means and standard deviations. Whole blood CsA was measured by radioimmunoassay with a monoclonal antibody, 3 hours (peak) and 12 hours (trough) after per os administration of the drug (Sandimmun® Sandoz Laboratories). ADO plasma levels were significantly higher in kidney transplant recipients than in chronic renal failure group (Mann-Whitney test: S = 5.5 P = 1.6 × 10⁻⁵) and than in controls (S = 8.5, P = 2.1 × 10⁻⁵). In non-CsA-treated kidney transplant recipients, ADO plasma levels were in the same range as those of controls. Finally, a high ADO plasma level was found in the two FK506-treated patients. Abbreviations are: CsA, cyclosporine A; ADO, adenosine; BUN, blood urea nitrogen concentration.

Blood samples

Sample collection and treatment have been described previously [27–29]. Briefly, total venous blood (3 ml) was drawn simultaneously with a stopping solution in Vacutainer tubes under vacuum (one sample per patient). With this method, the blood sample can be rapidly mixed with 10 ml of stopping solution, which prevents ADO uptake by BRC [26, 30, 31]. We checked that the mixed solution was correct by measuring hematocrits (mean values 44% ± 3; mean values of the hematocrits with stopping solution 17.6% ± 5). The stopping solution was composed of 0.2 mm dipyridamole, 4.2 mM Na₂EDTA, 5 mM EHNA, 79 mM AOPCP, heparin sulfate 1 IU/ml, and 0.9% NaCl. The sample plus stopping solution was centrifuged at 2500 g for 10 minutes and the supernatant was deproteinized by adding 2 ml of 70% perchloric acid before a second centrifugation (2,500 g for 10 minutes). The supernatant was lyophilized and redissolved in 1 ml of 50 mm sodium phosphate buffer (pH 4). The resulting solutions were filtered by centrifugation in a Millipore Ultrafree-MC 0.45 μm filter before being chromatographed.

High pressure liquid chromatography

The high pressure liquid chromatography (HPLC) technique has been described previously [27–29]. Briefly, plasma adenosine was assayed by reversed phase HPLC on Merck C18 columns (250 × 4 mm). The column was equilibrated with 50 mm sodium phosphate buffer (pH 4). The sample was injected and was eluted with a methanol gradient (0% to 46% methanol in 40 minutes) at a flow rate of 1 ml/min. ADO was identified by elution time and after incubation with adenosine deaminase, which increases the inosine peak and decreases the ADO peak [27]. ADO was quantified by comparing the peak areas given by known quantities.
of adenosine. In these conditions, the sensitivity threshold was 10 pm injected in 1 ml.

**In vitro experimental procedure**

To determine the interaction between adenosine deaminase and CsA, we mixed 1 ml aliquots of cell-free heparinized plasma obtained by centrifugation (2500 g for 10 min) from whole blood samples of six volunteers with 10 IU/ml of adenosine deaminase plus CsA (Sandimmun®; Sandoz Laboratories, France) at 250 or 500 ng/ml. Samples were incubated for 5 minutes at 37°C with continuous mixing by a Vortex® system, after which 1 nmol (in 10 μl) of ADO was added. The degradation of adenosine was stopped 5, 10, 15 and 30 seconds after ADP addition by using 3 ml of cold stopping solution (see above). Samples were immediately stored at −80°C before being chromatographed as described above.

**Adenosine uptake by red blood cells**

Samples of 1 ml of RBC were washed three times with 5 ml of 0.9% NaCl. After the last wash, 5 IU/ml of heparin was added and samples were mixed with 1 ml of a solution containing 0.9% NaCl, 2 μM CaCl₂, 1 μg of deoxycoformycin, and 0.5 mg of indomethacin. Deoxycoformycin was added to prevent any action of residual adenosine deaminase in the samples. Indomethacin was added to prevent ADO release by cells during centrifugation [22]. The same procedure as described above for plasma samples was then carried out. After centrifugation (1500 g for 10 min), the supernatants were placed at −80°C before being chromatographed.

**Statistical analysis**

The Mann-Whitney test was used to compare ADO plasma levels between controls and patients. An analysis of variance was used to compare ADO levels as a function of time in studies in vitro. We used the Spearman correlation coefficient for correlation studies.

**RESULTS**

**Adenosine plasma levels**

Adenosine plasma levels in CsA-treated KTR were significantly higher (mean 0.76 μM ± 0.27) than in the healthy subject group (mean 0.31 ± 0.13; Mann-Whitney test S = 8.5; P = 2.1 × 10⁻³) and than those in the chronic kidney failure group (Mann-Whitney, S = 5.5; P = 1.6 × 10⁻³). On the basis of serum creatinine, the chronic kidney failure group had the same renal function as the CsA-treated KTR group (Table 1). In the CsA-treated KTR group, CsA blood levels correlated both with ADO plasma levels (Spearman’s R = 0.8, P = 1.9 × 10⁻³, Fig. 1) and with mean blood pressure (Spearman’s R = 0.64, P = 4.2 × 10⁻²). ADO plasma levels in non CsA, non-FK506-treated KTR (KTR control group) were in the same range as those of the healthy subjects (mean 0.26; so 0.14). Finally, a high ADO plasma level was also found in the two patients under FK506.

**In vitro study**

In cell-free plasma, CsA had no effect on ADO degradation by adenosine deaminase, since the ADO half-life was not significantly modified in the presence of 250 or 500 ng/ml of CsA (analysis of variance P > 0.05 in both cases, Fig. 2).

In adenosine deaminase-free samples, ADO uptake by RBC in the presence of 500 or 1000 ng/ml (but not 250 ng/ml) of CsA was significantly lower than in controls (analysis of variance, P = 1.8 × 10⁻³, P = 1.2 × 10⁻⁴, and P > 0.05, respectively; Fig. 3). These CsA sample levels are in the range of those observed in KTR three hours after CsA per os administration (Table 1).

**DISCUSSION**

Our results demonstrate that the chronic administration of CsA or analog to kidney transplant recipients is accompanied by high plasma ADO levels. As all chromatographed samples were incubated with adenosine deaminase, the enzyme that metabolizes ADO into inosine, we can exclude that the high plasma ADO
levels observed result from an interference between CsA adducts and the ADO assay.

Because ADO has a very short half-life, elimination in urine is not a classical metabolic pathway [22, 32]. This probably explains why ADO plasma levels are not modified in the chronic kidney failure group. It appears that the increase in ADO plasma levels is induced by CsA administration for three reasons: (1) CsA inhibits ADO uptake by RBC and increases its half-life in vitro; (2) CsA blood levels are correlated with ADO plasma levels; and (3) even if there were too few non-CsA-treated KTR for statistical analysis, their ADO plasma levels were in the same range as controls. These findings show that the other immunosuppressive drugs (prednisone and azathioprine) are not involved in ADO release. Finally, CsA adducts are probably not implicated in the inhibition of the ADO uptake by RBC since this inhibition began only a few minutes after the in vitro incubation. However, even though CsA adducts have few metabolic effects [1], we cannot speculate on possible interactions between CsA adducts and ADO uptake by RBC in vivo.

In fact, ADO is a powerful immunosuppressor via its transformation into deoxyadenosine, which is then preferentially converted into the toxic compound deoxyadenosine triphosphate that inhibits T cell functions [5–7]. Thus, adenosine deaminase deficiency is accompanied by high ADO plasma levels and severe combined immunodeficiency [6–7]. By binding to intracytoplasmic cyclophilin [33], CsA inhibits interleukin II and interferon γ mRNA production in T cells [34]. However, ADO plasma level increase by CsA may be another mechanism that participates in the immunosuppressive effects of CsA.

The increase in ADO plasma levels may also participate in CsA-induced renal toxicity. Depending on its concentration, ADO can decrease or increase renal blood flow because it interacts both with high affinity A1 receptors inducing renal vasoconstriction and with low affinity A2 receptors inducing vasodilatation [35]. Contrary findings have been reported on the ability of ADO receptors antagonists to reverse the CsA-induced renal vasoconstriction. Some studies have shown that the ADO receptor antagonist theophylline could not reverse acute CsA-induced renal vasoconstriction [19, 36]. On the contrary, others in which CsA was chronically administered have shown that pentoxiphylline, a phosphodiesterase inhibitor that is also an adenosine antagonist [37], decreases CsA nephrotoxicity [38–40]. The later result suggests that the renal protective effects of pentoxiphylline are mediated by the blockade of ADO action. In fact, the contradictory results for ADO antagonists on CsA vascular effects may be explained by the fact that ADO antagonists have opposite effects in chronic and acute exposure to ADO [41].

It was reported that CsA can induce vasoconstriction directly by acting on smooth muscle [42] or via the autonomic nervous system [43]. This vasoconstriction may be responsible for oligemia-induced ADO release, since ADO is released by cells in cases of oligemia and/or tissue hypoxia, even minimally [22]. Finally, these high ADO blood levels resulting both from CsA-induced inhibition of ADO uptake by RBC and probably from oligemia-induced ADO release may play a role in CsA-induced renal vasoconstriction.

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REFERENCES

5. BAER HP, DRUMMOND GD: Physiological and regulatory function of adenosine and adenosine nucleotides, edited by BAER HP, DRUMMOND GD. New York, Raven Press, 1979
10. SULLIVAN BA, HAK LJ, FINN WF: Cyclosporine nephrotoxicity; studies in laboratories animals. Transplant Proc 17 (Suppl 1):145–154, 1985