Clinically unexpected cyclosporine levels using the ACMIA method on the RXL dimension analyser

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Abstract
Safe use of cyclosporine (CsA) in solid organ transplantation relies on regular whole-blood drug monitoring. Several promising immunoassays, e.g. the antibody-conjugated magnetic immunoassay (ACMIA) method, were developed and commercialized during recent years to compete with liquid chromatography coupled to tandem mass spectrometry, which remains the reference method but is labor-intensive. We describe the occurrence of interference in the monitoring of whole-blood CsA after transplantation when using the ACMIA method and discuss the potential mechanisms involved in such interference. Clinically unexpected results of whole-blood CsA require immediate reassessment by another technique to prevent the risk of CsA underdosage and graft rejection.

Keywords: ACMIA; cyclosporine; drug monitoring; transplantation; interference

Introduction
Cyclosporine (CsA) has been widely used as an immunosuppressant drug in solid organ transplantation for >30 years. Appropriate use of this narrow therapeutic index medication relies on regular whole-blood drug monitoring. Since the years 1990, several immunoassays have been developed and introduced to optimize the care of patients under CsA treatment [1].

However, it has been previously shown that some common measurements performed by immunoassays are inherently prone to analytical interference, generally due to the presence of so-called interfering antibodies [2] or endogenous cross-reacting compounds [3]. This kind of interference was described for several immunoassays—e.g. thyroid function tests [4] and cardiac biomarkers—with an approximative occurrence of falsely elevated or false-positive results of 0.4% [2].

Most available immunoassays for CsA monitoring are characterized by a certain extent of cross-reactivity with CsA metabolites, acceptable in clinical practice, and provide lower laboratory workload than liquid chromatography coupled with mass spectrometry (LC-MS/MS), together with good robustness and consistency in the results.

Here, we report a first case of major interference in the monitoring of whole-blood CsA in a heart–kidney transplant recipient when using the antibody-conjugated magnetic immunoassay (ACMIA) method.

Case presentation
A 58-year-old man underwent combined heart and kidney transplantation in August 1995 for Stage IV heart failure resulting from decompensated aortic valvulopathy and chronic kidney disease attributed to chronic glomerulonephritis. Baseline immunosuppression relied on CsA (Neoral; Novartis Pharmaceuticals, Basel, Switzerland), azathioprine and steroids, after rabbit anti-thymocyte glo-
bulines (ATG) (Thymoglobulin; Genzyme, Cambridge, MA) induction. Immediate post-operative course was uncomplicated. Creatinine decreased from 2.75 mg/dL at the time of transplantation to 1.0 mg/dL at Day 10. Repeated endomyocardial biopsies did not reveal signs of acute or chronic rejection. Further evolution was unremarkable except for the discovery of an immunoglobulin M monoclonal gammopathy in 1996, a percutaneous transluminal angioplasty with stenting of the left iliac artery in 2000 and of the right coronary artery in 2006. Repeated gout crisis led in February 2006 to the replacement of azathioprine by mycophenolate mofetil 500 mg t.i.d. in order to add allopurinol 150 mg o.d. At this time, other medications included CsA 35 mg b.i.d., methylprednisolone 3 mg o.d., hydrochlorothiazide 25 mg o.d., losartan 100 mg o.d., acetylsalicylic acid 100 mg o.d., bisoprolol 5 mg o.d., simvastatine 20 mg o.d. and pantoprazole 20 mg o.d.

This treatment remained unchanged during the following 4 years, except for the dose of CsA that was adapted to target whole-blood concentrations around 100–150 ng/mL, 12 h after the evening dose (C0). This regular therapeutic drug monitoring was performed using the ACMIA method run on a dimension RXL system (Siemens Healthcare Diagnostics, Deerfield, IL). This ACMIA method for CsA monitoring was introduced in our institution in 2005 with successful participation to external quality assessment schemes.

Adequate immunosuppressive management was achieved until June 2008, provided slight modifications in CsA daily dose (Figure 1). Between June 2008 and June 2009, the dose had to be reduced from 25 mg b.i.d. to 10 mg b.i.d., with no concomitant modification in regular therapy nor any change in diet. In October 2009, despite further reduction to 10 mg once daily, whole-blood CsA concentrations at 24 h remained unexpectedly high, at 214 ng/mL, inducing for the first time the suspicion of an interference with the assay. CsA was withdrawn and replaced by extended-release tacrolimus (Advagraf; Astellas Pharma, Staines, UK) to achieve tacrolimus trough levels of 7–10 ng/mL. Eighteen, 23 and 60 days after CsA withdrawal, whole-blood CsA concentrations were still at 225, 202 and 167 ng/mL, respectively. The hypothesis of interference with the immunoassay was subsequently confirmed by measuring CsA concentrations with another immunoassay, the CMIA (chemiluminescence microparticle immunoassay), on the Architect instrument (Abbott Diagnostic, Wiesbaden, Germany) [5] and by LC-MS/MS. CsA was undetected in the three samples by these two comparative methods. Additional confirmation was obtained by reintroducing a single 10-mg Neoral dose and measuring CsA concentrations 2 h later by ACMIA and LC-MS/MS. Concentrations of 206 and 40 ng/mL were obtained (Table 1). It is of interest to note that there is some proportionality between the results obtained with ACMIA and LC-MS/MS, as shown by comparing baseline and C2 results after a unique dose of 10 mg of Neoral (Table 1).

On repeated questioning, the patient signaled no change in his life habitus, no use of new medication or dietetic preparations, new exposition to animal proteins (through pets or via occupational exposition), no recent infection or immunization and no blood transfusion or treatment with monoclonal therapy. Complementary lab tests showed stable kidney graft function with a creatinine of 1.58 mg/dL (139 μmol/L), normal haemoglobin level (12.3 g/dL) and haematocrit (38%), no rheumatoid factor or antinuclear antibodies, and no further increase in serum IgM antibodies (334 mg/dL, normal < 230 mg/dL).

Fig. 1. Daily CsA measurement and whole-blood monitoring using ACMIA method on the RXL Dimension analyser.
In an attempt to identify the origin of the interference—possibly involving heterophile antibodies, three methods were used. First, serial dilution of patient’s whole blood (2- to 4-fold dilution factor) resulted in non-proportional and non-linear values, suggesting the presence of an interference. Secondly, pre-treatment with heterophile blocking tubes (HBT; Scantibodies Laboratories, Santee, CA) according to the manufacturer protocol failed to remove the interference, either on separated ethylene-diamine-tetraacetic acid (EDTA) plasma or on EDTA whole blood. Finally, the addition of an equal volume of polyethylene glycol (PEG 6000; 250 g/L in 0.05 mol/L phosphate buffer, pH 7.4, containing 0.5 g/L Triton X-100) to the EDTA plasma entirely suppressed the interference. Immunofixation performed on the pellets isolated after centrifugation showed that the vast majority of the immunoglobulins corresponded to IgG kappa.

**Discussion**

We present here the case of a heart and kidney transplant recipient in whom whole-blood CsA concentrations were falsely elevated as determined by the ACMIA method. Comparison to results obtained by an alternative immunoassay method and by the reference technique LC-MS/MS confirmed the suspected interference with ACMIA.

Many immunosuppressive drugs used in solid-organ transplantation, e.g. mainly calcineurin inhibitors, require regular therapeutic drug monitoring to assess efficacy, prevent rejection and avoid side effects or toxicity. Therapeutic CsA monitoring has been established as part of routine clinical management of transplant recipients for >25 years. LC-MS/MS is the reference method but remains limited to large centres because of both high cost equipment investment and experienced staff workload [1].

Several immunoassay methods (Cedia Plus, enzyme-multiplied immunooassay technique, monoclonal fluorescence polarization immunooassay (mFPIA), Incstar mRIA, ACMIA and recently CMA) have been developed and commercialized. Contrary to mFPIA that lacks specificity because of cross-reactivity with CsA metabolites [1], other immunoassays do correlate satisfactorily with LC-MS/MS.

ACMIA from Siemens Diagnostics is a newly developed fully automated assay, using a mouse capture antibody, for whole-blood CsA or tacrolimus monitoring, that requires no pre-treatment and allows decreased workloads and fast throughput—availability of the result <20 min after sample reception by the laboratory. The Dimension analyser mixes and lyses the whole-blood sample and then adds a CsA (or a tacrolimus) antibody/β-galactosidase conjugate. Magnetic particles coated with CsA (or tacrolimus) are added to bind free antibody–enzyme conjugates and are separated magnetically. The remaining supernatant containing the antibody–enzyme complex is then mixed with the substrate, and β-galactosidase catalyses the hydrolysis of chlorophenol red β-D-galactopyranoside to produce chlorophenol red that absorbs light at 577 nm. In two recent studies, whole-blood CsA and tacrolimus concentrations obtained with the ACMIA assay were highly correlated with alternative immunoassays [6, 7].

However, during the last few months, several cases of falsely elevated tacrolimus measurement using the ACMIA method were reported in liver [8, 9] or kidney [10, 11] transplant recipients. In a retrospective review of transplant patients with clinically unexpected tacrolimus levels using the ACMIA method, false-positive results were shown to occur in ~1% when compared to the LC-MS/MS reference method [12].

Such results are in fact not really surprising as it is now acknowledged that immunoassays—which fundamentally are in vitro immunological-binding reactions between the molecules to be measured and a reagent antibody—lack monospecificity [2]. Consequently, some endogenous heterophile antibodies—that is, human antibodies that may bind the animal antibodies used in an immunoassay, or other compounds, can interfere with the immunological assay and lead to erroneous results. Such antibodies can be produced in response to infection, recent immunization, treatment with monoclonal antibodies, blood transfusion, exposition to animal proteins or in the case of autoimmune disorders [2]. In patients reported with false-positive results for tacrolimus monitoring, the presence of heterophilic antibodies was demonstrated in one case [9] and the coexistence of auto-antibodies—such as rheumatoid factor [8] or anti-doubled stranded DNA antibodies [11]—was noted in two other reports. Interestingly, among 50 patients with significantly seropositive rheumatoid arthritis not given tacrolimus, 2 (4%) were positive for whole-blood tacrolimus determination using the ACMIA method on an RXL Dimension analyser, and sample pre-treatment with an HBT suppressed apparent tacrolimus concentrations [13]. A recent publication reports a similar interference with the tacrolimus the ACMIA assay due to an endogenous antibody present in the patient’s sample that recognized a unique epitope present on the antibody-enzyme (β-galactosidase) conjugate used in the ACMIA method [14]. The pre-treatment steps needed in most immunoassays (cellular and protein precipitation) are expected to remove such antibodies. Since the ACMIA method does not involve any pre-treatment phase, this method is potentially more affected by the presence of such antibodies.

Now, concerning CsA monitoring, the present case is to our knowledge the first reported case of positive interference using the ACMIA method on an RXL Dimension system. To determine the nature of the observed interference, we tested two anti-interfering procedures, including the commercially available HBT and PEG precipitation. PEG acts as an inert solvent sponge, reducing solvent availability and leading to protein precipitation; when applied to serum, PEG precipitation is relatively specific for immunoglobulins and immunoglobulin complexes. Abolition of the interference after PEG pre-treatment thus

**Table 1.** Whole-blood CsA concentrations using ACMIA method and liquid chromatography coupled to tandem mass spectrometry at baseline and 2 h (C2) after oral administration 10 mg of Neoral

<table>
<thead>
<tr>
<th>ACMIA (ng/mL)</th>
<th>LC-MS/MS (ng/mL)</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>173</td>
</tr>
<tr>
<td>C2</td>
<td>206</td>
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strongly supports the presence of heterophile antibodies, even if HBT did not influence the results. Indeed, although the underlying mechanism remains unknown, it was previously shown that HBT treatment can produce a spuriously high recovery in some hormone assays, possibly dependent on assay configuration, and the technique used to detect heterophile antibody interference was shown to be specific to each assay method [15]. Moreover, in our current CsA assay and contrary to endocrine biomarkers affected by heterophile antibodies, most of the analyte is located in red blood cells; this may also contribute to the divergent results observed between the HBT and the PEG methods. The putative interfering role of monoclonal IgM antibodies remains elusive as their serum concentrations have been unchanged for many years and as the pellets obtained after pre-treatment with PEG 6000 predominantly contained IgG kappa antibodies. Previous reports have demonstrated that the use of rabbit anti-thymocyte globulins in transplant recipients is associated with the development of serum anti-rabbit antibodies [16] potentially leading to clinically significant interferences [17]. The putative immunization against rabbit ATG in our patient and its role in the observed interference remains hypothetic, and the capture antibody used in the ACMIA assay is from a different species (mouse). Further investigations are required to determine whether the use of rabbit ATG in solid organ transplantation is associated with the development of such antibodies interfering with the monitoring of CsA performed with the ACMIA method. Fortunately, the outcome of our patient’s heart and kidney grafts was favourable, with no documented rejection, while probably underdosed in CsA for weeks or months.

Clinicians involved in the follow-up of transplant recipients and laboratory physicians should thus be aware of such interference in the measurement of whole-blood CsA using the ACMIA method, leading to falsely elevated or false-positive results. Clinically unexpected results of CsA using the ACMIA method, leading to falsely elevated concentrations of our patient formed with the ACMIA method. Fortunately, the outcome in whole blood with the RXL dimension analyzer: a useful tool for immunosuppressive drug monitoring. Transplant Proc 2009; 41: 707–709
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Conflict of interest statement. None declared.

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