Background: Graft survival depends on adequate immunosuppression. To evaluate the effect on the immune system of immunosuppressive therapies using calcineurin inhibitors (CNIs), several pharmacodynamic indices have been proposed to complement pharmacokinetic data. In this preliminary study we compared some of these parameters during combined immunosuppressant therapies.

Methods: We treated 65 stable renal transplant recipients with cyclosporin A (CsA; n = 11549), 16), tacrolimus (TRL; n = 10), CsA + mycophenolate mofetil (MMF; n = 14); TRL + MMF (n = 13), and MMF (n = 12). Twelve nontreated healthy controls were also included. Calcineurin activity (CNA) in peripheral blood mononuclear cells was measured using 32P-labeled peptide. Interleukin-2 (IL-2) and interferon-γ production in phytohemagglutinin-activated whole blood were measured at 0 and 2 h postdose. The areas under the curves, c_{min}, c_{max}, and concentration at 2 h (c_{2h}) were also measured.

Results: We found no differences in CNA between groups receiving CNIs alone or combined with MMF [median (25th–75th percentiles)]; CsA_{2h}, 3.87 (3.00–6.85)% alkaline phosphatase (AP); CsA + MMF_{2h}, 3.90 (1.78–5.19)% AP; TRL_{2h}, 5.68 (3.02–16.00)% AP; TRL + MMF_{2h}, 11.80 (4.05–14.63)% AP. In vitro IL-2 production was significantly lower in the groups receiving combined therapy than in groups receiving CNIs alone [median (25th–75th percentiles)]; CsA_{2h}, 276.52 (190.41–385.25) ng/L; CsA + MMF_{2h}, 166.48 (81.06–377.01) ng/L (P < 0.001); TRL_{2h}, 249.34 (127.48–363.50) ng/L; TRL + MMF_{2h}, 122.13 (51.02–180.00) ng/L (P < 0.001). The correlations (r) between c_{2h} and CNA 2 h postdose were as follows: CsA, r = −0.74; CsA + MMF, r = −0.84; TRL, r = −0.70; TRL + MMF, r = −0.70 (P < 0.001 in all cases).

Conclusions: The measurement of CNA may be of help in following the effect on the immune system of CNI treatments, even in combined therapies, but does not reflect the additional effect of MMF. In contrast, IL-2 in vitro production reflects the effect of both MMF and CNIs.

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Current standard immunosuppressive therapy protocols are generally highly effective in preventing acute rejection. However, long-term immunosuppressive treatment has substantial adverse effects, and its efficacy in preventing chronic rejection is poor. For this reason there is growing interest in evaluating the efficacy and safety of lower toxicity immunosuppressive therapies such as combinations of calcineurin inhibitors (CNIs) at low doses and antimetabolites such as mycophenolate mofetil (MMF).

The measurement of blood concentrations of immunosuppressants provides only an indirect evaluation of the degree of immunosuppression attained in an individual patient (1, 2). When combined treatments are used, the biological impact may be higher than that predicted by the individual blood concentrations of each immunosup-

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Nonstandard abbreviations: CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; CNA, calcineurin activity; PD, pharmacodynamic; CsA, cyclosporin A; TRL, tacrolimus; IL, interleukin; IFN, interferon; FKBP, FK-binding protein; PK, pharmacokinetic; AUC, area under the curve; NHC, healthy control; DTT, dithiothreitol; PHA, phytohemagglutinin; TFA, trifluoroacetic acid; AP, alkaline phosphatase; PBMC, peripheral blood mononuclear cell; and MPA, mycophenolic acid.
pressant. Thus the measurement of several markers has been proposed to assess the impact of immunosuppressants on the immune systems of individual patients. Various approaches have been proposed for the pharmacodynamic (PD) monitoring of immunosuppressants: One is evaluation of the activity of the specific target enzyme, such as calcineurin activity (CNA) for cyclosporin A (CsA) and tacrolimus (TRL) (3–5), inosine monophosphate dehydrogenase for MMF (6–8), and P70S6 kinase activity for sirolimus (I, 9). A second approach is the evaluation of an intermediate step in the action mechanism: for example, the measurement of interleukin-2 (IL-2) and interferon-γ (IFN-γ) production in vitro in whole blood for CsA and TRL (10), and DNA duplication for MMF and sirolimus (7, 11). A third possibility is the measurement of collateral markers modified by the presence of the immunosuppressant, such as the T-lymphocyte surface antigens CD25, CD71, and CD154 (12, 13).

CsA and TRL are widely used to prevent rejection in allotransplantation. Both drugs have the same target: the serine/threonine phosphatase calcineurin (CN), or PP2B. One important substrate of this phosphatase is the nuclear factor of activated T cells. Nuclear factor of activated T cells remains in the cytoplasm when it is inactive. It is activated by dephosphorylation effected by CN and migrates to the nucleus, where it participates in the transcription of genes necessary for lymphocyte expansion, such as IL-2, IFN-γ, IL-4, IL-3, and tumor necrosis factor-α (14). To inhibit CNA, CsA and TRL must bind to the immunophilins present in the cytoplasm of T lymphocytes: cyclophilins in the case of CsA and FK-binding proteins (FKBPs) in the case of TRL. CsA–cyclophilin (CsA–cyclophilin A) and TRL–FKBP (TRL–FKBP12) complexes inhibit ~80–90% of CNA (15–17).

To our knowledge, no data on the correlation between CNA and IL-2 or IFN-γ production have been reported in stable renal patients receiving CsA or TRL alone or in combined therapy with MMF.

The aims of this study were (a) to determine the correlation between the pharmacokinetic (PK) parameters [c_{min}, c_{max}, c_{2h}, and area under the curve (AUC)] for CsA and TRL and the different PD indices proposed (CNA and IL-2 and IFN-γ production), and (b) to assess the importance of combined therapies on the measurement of CNA and IL-2 and IFN-γ production. Establishing additional PD indices to measure the biological impact of immunosuppressants can be of help in the introduction of new immunosuppressant combinations, in the introduction of lower-than-standard doses, and in the study of individual variability in certain special cases.

Patients and Methods

Patients

This was a nonrandomized (non-placebo-controlled) trial. The study was approved by the Institutional Ethical Review Board of Hospital Clinic of Barcelona, and informed consent was obtained from all participants. Sixty-five stable renal transplant patients (39 males) were included in the study. The mean (SD) age was 58 (14) years, with a mean time since renal transplantation of 56 (14) months. Patients were divided into five groups according to their maintenance immunosuppression treatment: CsA monotherapy (n = 16), TRL monotherapy (n = 10), CsA + MMF (n = 14), TRL + MMF (n = 13), and MMF monotherapy (n = 12). The immunosuppressive drug treatment also included corticoids. All patients were stable, with acceptable renal function [mean serum creatinine 16.0 (4.5) mg/L], and with no episodes of acute rejection in the previous 6 months. No patient had clinical symptoms of nephrotoxicity. Immunosuppressive therapy had been used for more than 7 years in the CsA monotherapy group and for more than 5 years in the other groups. The mean (SD) doses were as follows: CsA monotherapy, 2.60 (0.90) mg · kg^{-1} · day^{-1}; TRL monotherapy, 0.08 (0.02) mg · day^{-1}; MMF monotherapy, 2.00 g/day; CsA + MMF therapy, 1.93 (0.50) mg · day^{-1} and 2.00 g/day, respectively; and for TRL + MMF, 0.09 (0.04) mg · day^{-1} and 1.00 g/day, respectively.

We recruited 12 healthy individuals [NHC; mean age 43 (8) years] as a control group for basal determinations.

Reagents

Protein kinase (3’:5’-cyclic AMP-dependent, from bovine heart); okadaic acid; the protease inhibitors aprotinin, leupeptin, and soybean trypsin; phenylmethylsulfonyl fluoride; dithiothreitol (DTT); MES; Tris; phytohemagglutinin (PHA); and trifluoroacetic acid (TFA) were purchased from Sigma. Acetonitrile was provided by Scharlau. CN substrate was obtained from Neosystem S.A. (Groupe SNPE) and [γ-^{32}P]ATP (10 Ci/L) was purchased from Nuclear Ibérica S.A. C_{18} solid-phase extraction columns (Sep-Pak) were from Supelco®. AG50W-X8, 200–200 mesh, cation-exchange resin was obtained from BioRad Laboratories. Alkaline phosphatase (AP; 20 U/μL) was from Boehringer Mannheim. OptiPhase “HiSafe” 2 was from Wallac Scintillation Products. CsA and TRL were kindly supplied by Novartis Farmacéutica S.A. (Barcelona, Spain) and Fujisawa Pharmaceutical Co, Ltd. (Osaka, Japan), respectively. Trichloroacetic acid and K_{2}HPO_{4} were provided by Merck. Human IL-2 and human IFN-γ immunoassays were obtained from Immuno-technet.

Cell Preparation

Human peripheral blood mononuclear cells (PBMCs) were obtained from the mononuclear cell layer of the Ficoll-Hyphaque gradient. The remaining red blood cells were lysed during a short incubation period (10 s) in water followed by addition of an equal volume of phosphate-buffered saline (2× concentrate). Washed and pelleted PBMCs (6 × 10^{6}) were lysed with 0.3 mL of hypotonic lysis buffer containing protease inhibitors [50 mmol/L Tris (pH 7.5), 0.1 mmol/L EGTA, 1 mmol/L EDTA, 0.5 mmol/L DTT, 50 mg/L phenylmethylsulfonyl
fluoride, 50 mg/L soybean trypsin, 5 mg/L leupeptin, and 5 mg/L aprotinin]. Lysis was facilitated by three rounds of freezing in liquid nitrogen followed by thawing at 30 °C. Cellular debris was sedimented by centrifugation at 4 °C for 10 min at 12 000g, and the clear supernatant was frozen and stored for at most 2 weeks in liquid nitrogen without significant loss of CN activity (18).

PHOSPHORYLATION OF SYNTHETIC PePTIDE
A 19-amino-acid peptide derived from cAMP-dependent kinase regulatory subunit type II was phosphorylated in vitro and used as a substrate to measure CNA. The 19-amino-acid peptide (19mer) sequence was Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu. The lyophilized peptide was dissolved in water to a concentration of 3.30 mmol/L. Phosphorylation of the serine residue with [γ–32P] ATP was performed with the catalytic subunit of cAMP-dependent kinase. The kinase reaction contained 50 μL of buffer A [40 mmol/L MES (pH 6.5), 0.40 mmol/L EGTA, 0.80 mmol/L EDTA, 4 mmol/L MgCl2, 0.10 mmol/L CaCl2, and 0.10 g/L bovine serum albumin], 12 μL of [γ–32P] ATP (10 Ci/L), 9 μL of 3.30 mmol/L peptide, 19 μL of H2O, and 10 μL of 160 mg/L protein kinase. After the reaction mixture had incubated for 1 h at 30 °C, we added 900 μL of H2O.

The phosphorylated peptide was purified by Sep-Pak C18 chromatography. Columns were prepared using syringes to apply 3 mL of 300 mL/L acetonitrile in 1 g/L TFA followed by 5 mL of 1.0 g/L TFA. The contents of the kinase reaction were then slowly applied to the column. The column was washed with 400 mL of TFA to remove unincorporated ATP. The peptide was then eluted by addition of five 0.5-mL volumes of 300 mL/L acetonitrile in 1.0 g/L TFA. The radiolabeled peptide fractions were pooled and evaporated under argon gas, resuspended with 2 mL of buffer 1 [20 mmol/L Tris-HCl (pH 8), 100 mmol/L NaCl, 6 mmol/L MgCl2, 0.10 mmol/L CaCl2, 0.50 mmol/L DTT, and 0.10 g/L bovine serum albumin], and stored at −20 °C (18).

The interassay CV for peptide labeling was 23% and was assessed by phosphorylating the synthetic peptide on different days with the same batch of [γ–32P]ATP and protein kinase. The intraassay CV of the phosphorylation assay was 5.6% and was assessed by performing five replicates of the phosphorylation assay on the same day.

PHOSPHATASE ASSAY (CNA MEASUREMENT)
CNA was measured in treated patients and in the NHC group in the morning predose and 2 h postdose.

Hypotonic lysates of PBMCs were evaluated for their ability to dephosphorylate a 32P-serine-labeled 19-amino-acid peptide substrate (19mer) in the presence of okadaic acid, a phosphatase type 1 and 2A inhibitor, as described previously by Fruman et al. (18). Background phosphatase 2C activity (CsA- and okadaic acid-resistant activity) was determined and subtracted from each sample, with the assay performed in the presence and absence of excess added CsA or TRL. The remaining phosphatase activity was taken as CNA (PP2B).

Assays were performed in a final volume of 60 μL, in four different tubes. In all tubes we added 20 μL of PBMC lysates and 20 μL of 32P-labeled phosphopeptide (final concentration, 5 μmol/L) as substrate. In addition, in tube 1 we added 20 μL of buffer 1, in tube 2 we added 20 μL of buffer 2 (buffer 1 containing 500 nmol/mL okadaic acid), in tube 3 we added 20 μL of buffer 3 (buffer 2 containing 10 μmol/mL CsA), and in tube 4 we added 20 μL of buffer 4 (buffer 2 containing 100 μg/mL TRL). Buffer 4 has not been described before and was added to better evaluate samples from patients treated with TRL. We found no significant differences between buffers 3 and 4. The results were expressed in relationship to classic buffer 3. In addition, we measured the total activity of the substrate by counting 20 μL in a Beckman scintillation counter on the day of the assay, by measuring the spontaneous release via incubation of the substrate without lysate, and the capacity of AP (20 U) to dephosphorylate the substrate (maximum dephosphorylation).

The mixture was incubated for 30 min at 30 °C, and the reaction was stopped by the addition of 0.8 mL of 100 mmol/L K2HPO4 containing 50 g/L trichloroacetic acid. We then added 200 μL of cation-exchange resin (AG50W-X8, 100–200 mesh) and shook the suspension for 30 min at room temperature.

Cation-exchange resin was prepared in a batch procedure as follows. We suspended 2 g of dry resin was in 50 mL of water. After settling, the water was decanted and replaced with 5 mL of 1 mol/L NaOH. After mixing and settling, the supernatant was again decanted and replaced with 10 mL of 1 mol/L HCl. After mixing and settling, the supernatant was again decanted, and the resin was washed with 20 mL of water. After removal of this supernatant, the resin was suspended in 2.2 mL of water and stored at 4 °C.

After the incubation, we centrifuged for 2 min at 12 000g. The released inorganic phosphate contained in the supernatant (500 μL) was measured by scintillation counting (18).

The initial results indicated the need to introduce an internal value to minimize the variability among different batches of 32P-labeled synthetic peptide and the radioactive decay. The internal value was defined as the capacity of AP to dephosphorylate the synthetic peptide, and it was considered to be the maximum dephosphorylation that could be observed with the peptide batch in use. The internal value was introduced in all remaining experiments. The results for CNA were expressed as: percentage of AP = 100 (cpm released by the sample)/cpm released by the AP). CsA-resistant phosphatase values (standard method) or TRL-resistant phosphatase values (buffer 4) were subtracted.
from the phosphatase activity in presence of okadaic acid and CNA (PP2B) was expressed as percentage of AP.

The interassay and intraassay CVs for measurement of CNA were 8% and 5%, respectively. CNA was assessed by individuals who were blinded to the treatment allocation of the patient.

MEASUREMENT OF IL-2 AND IFN-γ PRODUCTION

IL-2 and IFN-γ production was measured in treated patients and in the NHC group in the morning predose and 2 h postdose. IL-2 and IFN-γ production was assessed by individuals who were blinded to the treatment allocation of the patient.

We measured the IL-2 and IFN-γ production in 950 μL of whole blood incubated with 50 μL of PHA (1 g/L) and shaken for 5 h at 37 °C. At the end of the incubation, the samples were centrifuged in an Eppendorf microcentrifuge for 2 min, and the supernatant was removed and stored at −80 °C until assayed. IL-2 and IFN-γ concentrations were measured by ELISA. The detection limits were 3 ng/L for IL-2 and 80 IU/L for IFN-γ.

MEASUREMENT OF BLOOD CONCENTRATIONS OF CsA AND TRL

CsA concentrations in whole blood were determined by Emit with a specific monoclonal antibody, on a Cobas Mira automated analyzer (Dade-Behring) as described previously (19). TRL monitoring was carried out by MEIA assay with an IMx analyzer (Abbott) (20). Blood samples for determining the CsA and TRL blood concentration-time curve AUCs were drawn predose (cmin) and at 1, 2, 4, 6, 8, 10, and 12 h after the morning dose from stable, treated renal patients. AUCs were calculated by the linear trapezoidal rule.

MEASUREMENT OF PLASMA CONCENTRATIONS OF MYCOPHENOLIC ACID

Plasma concentrations of mycophenolic acid (MPA) were analyzed by a validated HPLC-ultraviolet detection method described elsewhere (21, 22). The total run time was 12 min. The working range for MPA was 0.10–50 μg/L, and the within- and between-run CVs ranged from 4.5% to 9.7%.

International CsA, TRL, and MPA Testing Scheme

Samples were also analyzed as external controls (from D.W. Holt, European Quality Control, London, UK).

STATISTICS

Unless specified, all results are expressed as the median and the 25th–75th percentiles or the confidence interval. In some graphs, the 25th and 75th percentiles are shown. Statistical differences between groups were assessed using the nonparametric Mann–Whitney test. Differences are indicated on the graphs with an asterisk. Correlations between variables were assessed by another nonparametric test, the Spearman test (ρ), with SPSS statistical software (SPSS Inc.). P < 0.05 was considered significant.

Results

PK PROFILES OF THE TREATED PATIENTS

The PK parameters studied were cmin, c2, cmax, and AUC. The results were as expected according to doses received. Note that MMF doses in the TRL+MMF group were lower than for the other groups; as a consequence, the PK parameters are also reduced (Table 1).

CNA MEASUREMENTS IN VITRO

Measurement of CNA was validated in vitro. PBMCs from healthy donors were cultured in the presence of

![Table 1. PK profiles of treated patients.](image)
increasing concentrations of CsA (0–400 μg/L) or TRL (0–50 μg/L). After 24 h, cells were lysed, and the CNA was evaluated. The results obtained showed inhibitions >75% when the CsA concentration in cultures was >100 μg/L and the concentration of TRL was >12.50 μg/L (Fig. 1). Inhibitory concentrations may differ in vivo because of a clear difference between the free fraction of the immunosuppressant in biological fluids and in culture medium.

CNA MEASUREMENTS IN PATIENTS

CNA was evaluated at 0 and 2 h postdose in the PBMCs of stable renal transplanted patients treated with CsA, TRL, MMF, CsA+MMF, and TRL+MMF (Fig. 2).

The median (25th–75th percentiles) CNA was 37.45 (21.32–56.15)% AP in the NHC group and 24.20 (17.72–35.58)% AP in the MMF group. These values were significantly lower in the CsA group than in NHC or MMF group, both predose [0 h; 6.42 (5.47–10.01)% AP] and 2 h postdose [3.87 (3.00–6.85)% AP; P < 0.01; Fig. 2A]. We observed similar values in the CsA+MMF group at 0 h [6.41 (4.88–12.22)% AP] and 2 h [3.90 (1.78–5.19)% AP; Fig. 2A]. The CNA was significantly lower in the TRL group than in the NHC and MMF groups [0 h, 8.66 (6.14–23.94)% AP; 2 h, 5.68 (3.02–16.00)% AP; P < 0.01; Fig. 2B] and the TRL+MMF group [0 h, 18.53 (7.63–26.62)% AP; 2 h, 11.80 (4.06–14.63)% AP; P < 0.01], although TRL patients displayed more variability than CsA patients. We found no significant differences in CNA between patients treated with CNIs alone and those treated with CNIs+MMF (Fig. 2).

IL-2 AND IFN-γ PRODUCTION IN WHOLE BLOOD FROM PATIENTS

IL-2 and IFN-γ production after in vitro stimulation of whole blood with PHA was evaluated in the study groups (Figs. 3 and 4). Results showed considerable variability in IL-2 production in the NHC and MMF groups. IL-2 production was lower 2 h postdose in all groups other than the NHC group (Fig. 3). The production of IL-2 postdose in the CsA or TRL+MMF groups [median (25th–75th percentiles), 166.48 (81.06–377.01) ng/L and 122.13 (51.02–180.00) ng/L, respectively] was lower than in the CsA- or TRL-alone groups [median (25th–75th percentiles), 276.52 (190.41–385.25) ng/L (P < 0.001) and 249.34 (127.48–363.50) ng/L (P < 0.001) respectively; Fig. 3]. We observed no predose differences in IFN-γ production between the control group and patients treated only with CNIs [median (25th–75th percentiles), 22.39 (9.78–34.34) IU/mL vs 29.25 (25.55–31.90) IU/mL]. Postdose, blood from the CsA, TRL, and TRL+MMF groups showed clear inhibition compared with blood from the NHC group (Fig. 4).

PD AND PK CORRELATIONS

The correlation between the classic PK parameters and PD indices was studied with the nonparametric Spearman test (p). P < 0.05 was considered significant (Table 2).

There was a significant inverse correlation (r > 0.7) between CNA 2 h postdose and the AUC for all groups studied and for CNA 2 h postdose and c2h, especially in the CsA and CsA+MMF groups.

IL-2 production 2 h postdose and c2h also were significantly inversely correlated in the CsA, CsA+MMF, and TRL+MMF groups, as were IL-2 production 2 h postdose and AUC in the TRL+MMF group.

There was no correlation between IFN-γ and cmin in any group. In contrast, IFN-γ production was signifi-

**Fig. 1.** Measurement of CNA in PBMCs (6 × 10⁶) from healthy volunteer, cultured in vitro for 24 h with CsA (0–400 μg/L) or TRL (0–50 μg/L).

After 24 h cells were disrupted with hypotonic buffer and the CNA was evaluated. The results are expressed as cpm/10⁶ cells.

**Fig. 2.** CNA in transplanted patients treated with CsA (n = 16), MMF (n = 12), CsA+MMF (n = 14), TRL (n = 10), and TRL+MMF (n = 13) and in NHCs (n = 12).

Quartiles 25 and 75 (boxes) and values <1.5 interquartile range (error bars) are shown. ■, 0 h predose; □, 2 h postdose. *, P < 0.05 compared with the NHC group. The results are expressed as % AP.
cantly inversely correlated with \( c_{2h} \) and AUC in the CsA group (Table 2).

To identify the redundancy among PD indicators, we studied the correlations between the pharmacodynamics at different times and the various PD indicators (Table 3). We found a strong correlation between CNA 0 h predose and CNA 2 h postdose in all groups (\( r = 0.93 - 0.96 \)). We also found a significant correlation between IL-2 production 0 h predose and IL-2 production 2 h postdose in the CsA groups (\( r = 0.92 - 0.96 \)) and the TRL groups (\( r = 0.75 - 0.82 \); Table 3).

**Discussion**

This study was designed to evaluate the potential usefulness of different PD assays, initially described for the evaluation of CsA treatment, in the evaluation of TRL treatment and of combined therapies with MMF.

This study included PD markers such as CNA in PBMCs and IL-2 and IFN-\( \gamma \) production in whole blood along with classic PK parameters, and compared them in stable renal transplant patients treated with CsA or TRL alone or in combination with MMF.

We decided to measure CNA in PBMCs and not in whole blood, as other authors have done (23), for several reasons: Our main interest was the impact of the drug on enzyme activity inside the lymphocytes, which are the main producers of relevant interleukins involved in the alloresponse. This impact probably depends on the equilibrium between uptake, cyclophilin binding, P-glycoprotein-dependent excretion, and probably other factors re-

### Table 2. Correlations between PD indices and PK parameters.

<table>
<thead>
<tr>
<th></th>
<th>CsA</th>
<th>CsA+MMF</th>
<th>TRL</th>
<th>TRL+MMF</th>
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<tbody>
<tr>
<td>CNA at 0 h vs ( c_{min} )</td>
<td>( r ) -0.675</td>
<td>-0.811</td>
<td>-0.580</td>
<td>-0.579</td>
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<td></td>
<td>( P ) &lt;0.001</td>
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<td>0.006</td>
<td>0.002</td>
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<tr>
<td>CNA at 2 h vs ( c_{2h} )</td>
<td>( r ) -0.735</td>
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<td>-0.692</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>CNA at 2 h vs AUC</td>
<td>( r ) -0.754</td>
<td>ND</td>
<td>-0.803</td>
<td>-0.836</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>ND</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-2 at 0 h vs ( c_{min} )</td>
<td>( r ) -0.492</td>
<td>-0.564</td>
<td>-0.537</td>
<td>-0.553</td>
</tr>
<tr>
<td></td>
<td>( P ) 0.011</td>
<td>0.005</td>
<td>0.018</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-2 at 2 h vs ( c_{2h} )</td>
<td>( r ) -0.701</td>
<td>-0.784</td>
<td>-0.571</td>
<td>-0.806</td>
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<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>&lt;0.001</td>
<td>0.011</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-2 at 2 h vs AUC</td>
<td>( r ) -0.681</td>
<td>ND</td>
<td>-0.656</td>
<td>-0.772</td>
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<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>ND</td>
<td>0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IFN-( \gamma ) at 0 h vs ( c_{min} )</td>
<td>( r ) -0.246</td>
<td>-0.013</td>
<td>-0.296</td>
<td>-0.394</td>
</tr>
<tr>
<td></td>
<td>( P ) 0.235</td>
<td>0.952</td>
<td>0.233</td>
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<tr>
<td>IFN-( \gamma ) at 2 h vs ( c_{2h} )</td>
<td>( r ) -0.725</td>
<td>-0.399</td>
<td>-0.456</td>
<td>-0.618</td>
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<td>0.050</td>
<td>0.004</td>
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<tr>
<td>IFN-( \gamma ) at 2 h vs AUC</td>
<td>( r ) -0.737</td>
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<td>0.006</td>
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</tbody>
</table>

* A nonparametric Spearman test \( (p) \) was applied.

ND, not done.
that the nadir of CNA in PBMCs was at 3 h postdose and decided to use whole blood because with the technique the environment was maintained throughout the assay time (5 h). This practice is currently used to adjust CsA concentrations in clinical practice.

CsA is feasible with the technique described by Fruman et al. (24). Moreover, the preparation method used a buffer with TRL in the phosphatase assay (buffer 4; 10%) of the synthetic peptide, which in our opinion increases the accessibility to the active site of the synthetic peptide used (27). Othn et al. have noted that the presence of MMF in CNI therapies, especially postdose, induces a decrease in variability in the CNI-treated groups. The variability of CNA in TRL-treated patients was lower than that in the CsA-TRL group, and the variability in the CNI-treated groups was lower than that in the NHC group (83% and 77%, respectively, at 0 h; 90% and 85% at 2 h postdose). This is probably attributable to the accumulation of certain immunophilins in the various compartments in the lymphocyte. In other words, we have altered the probability of the formation of the immunosuppressant–immunophilin active complex (CsA–cyclophilin and TRL–FKBP). However, when we used a buffer with TRL in the phosphatase assay (buffer 4; described in Materials and Methods) there was no significant change in the results.

In combined therapies with CNIs and MMF, the CNA measurement does not directly reflect the additional immunosuppressive effect of MMF. The measurement of IL-2 production, in contrast, seems to reflect the effect provided by the presence of MMF in CNI therapies, especially postdose. In the NHC group, IL-2 production was highly variable. Variability in the CNI-treated groups was lower, especially postdose. Because of the short in vitro culture time used, IL-2 production depended on the number of preactivated cells present in vivo in each individual. The decrease in variability in the CNI-treated patients was probably attributable to the inhibitory effect of MMF on clonal expansion of activated lymphocytes. The decrease in the number of active lym-

| Table 3. Correlations between PD indices.* |
|-------------------------------|----------------|----------------|----------------|----------------|
|                               | CsA   | CsA+MMF | TRL   | TRL+MMF |
| CNA at 0 h vs CNA at 2 h      |       |         |       |         |
| r                             | 0.930 | 0.936   | 0.962 | 0.940  |
| P                             | <0.001| <0.001  | <0.001| <0.001  |
| IL-2 at 0 h vs IL-2 at 2 h    |       |         |       |         |
| r                             | 0.918 | 0.958   | 0.747 | 0.824  |
| P                             | <0.001| <0.001  | <0.001| <0.001  |
| IFN-γ at 0 h vs IFN-γ at 2 h  |       |         |       |         |
| r                             | 0.674 | 0.819   | 0.884 | 0.827  |
| P                             | <0.001| <0.001  | <0.001| <0.001  |
| CNA at 2 h vs IL-2 at 2 h     |       |         |       |         |
| r                             | 0.426 | 0.792   | 0.709 | 0.518  |
| P                             | 0.030 |         | 0.001 | 0.011  |
| CNA at 2 h vs IFN-γ at 2 h    |       |         |       |         |
| r                             | 0.525 | 0.299   | 0.454 | 0.456  |
| P                             | 0.007 | 0.166   | 0.050 | 0.043  |
| IL-2 at 2 h vs IFN-γ at 2 h   |       |         |       |         |
| r                             | 0.618 | 0.545   | 0.402 | 0.565  |
| P                             | 0.001 | 0.011   | 0.110 | 0.012  |

* A nonparametric Spearman test (r) was applied.
phocytes, rather than a direct effect of MMF on CNA or in the production of IL-2 and IFN-γ, was probably responsible for the decrease in production.

IFN-γ production is highly variable in this population. There was a lack of correlation between c min and IFN-γ at 0 h, indicating that IFN-γ is not a useful marker for these studies.

IL-2 production was lower in the CsA+MMF group than in the CsA-alone group at 2 h. These differences cannot be explained by differences in the CsA concentrations: there was no significant differences in CsA dose [1.93 (0.50) vs 2.60 (0.90) mg·kg⁻¹·day⁻¹, respectively]. We found only small difference in the c min values [median (25th–75th percentiles), 61.50 (39.50–92.50) vs 93.00 (80.75–128.25) μg/L, respectively], and some differences in the c max were not statistically significant. No differences were evident in the CsA concentrations at 2 h postdose [median (25th–75th percentiles), 494.50 (251.00–654.25) and 481.00 (318.75–561.75) μg/L, respectively]; therefore, the presence of MMF seems to be responsible for the additional inhibitory effect on IL-2 production seen at 2 h in the CsA+MMF group.

IL-2 production was lower in the TRL+MMF group than in the TRL-alone group at 2 h. Those differences cannot be explained by differences in the TRL concentrations: neither the doses nor c min showed significant differences. The c₂₁ was even lower in the TRL+MMF group than in the TRL-alone group [median (25th–75th percentiles), 13.50 (11.70–17.10) and 21.90 (10.35–33.15) μg/L, respectively]; therefore, the presence of MMF seems to be responsible for the additional inhibitory effect on IL-2 production seen at 2 h in the TRL+MMF group as well.

These two results suggest that the addition of MMF to CNI therapies increases the inhibition of IL-2 production, although it does not directly affect CNA. The reason could be that reducing the clonal expansion of activated T cells reduces the number of cells ready to produce IL-2 in our in vitro assay.

Several strategies have been proposed in the past to monitor the pharmacodynamics of CNI immunosuppressants. Here we compared some of them in monotherapy and combined therapies to identify the most useful ones to monitor the biological impact of new combinations of immunosuppressants, different doses from those considered standard, or the study of problematic patients.

In our study, the PD indicator with the best correlation with AUC and c₂₁ was CNA, followed by IL-2 production. In general, IFN-γ had the poorest correlation with PK parameters; the correlation was particularly poor between IFN-γ₀ h and c min. IL-2 production, however, seems to reflect the additional immunosuppressive effect introduced by MMF in combined therapies. This additional effect was not detected by CNA determination.

There was a very good correlation with CNA at 0 and 2 h for both CsA and TRL and with IL-2 at 0 and 2 h, especially for CsA.

In conclusion, the measurement of CNA may be a good predictor of the immunosuppression caused by CsA or TRL monotherapies, whereas IL-2 production is potentially more useful for monitoring combined therapies comprising CNIs and MMF.

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References

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