Inhibition of Stimulated Interleukin-2 Production in Whole Blood: A Practical Measure of Cyclosporine Effect

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Background: Prediction of cyclosporine (CSA) efficacy and toxicity in individual patients is difficult. There is no practical, biologically relevant, pharmacodynamic measure of CSA effect. A major effect of CSA is to decrease interleukin-2 (IL-2) production; however, measurement of this effect in isolated lymphocytes as a marker of response to CSA has been problematic.

Methods: CSA inhibition of phytohemagglutinin-P (PHA)-stimulated IL-2 production, measured by ELISA, was studied ex vivo in whole blood drawn before, and after subjects received 4 mg/kg oral CSA.

Results: Four hours after CSA was administered, the mean (± SD) CSA concentration was 702 ± 196 μg/L and PHA-stimulated IL-2 production decreased by 68.7% ± 17.2% (P < 0.0001; n = 17). Twenty-four hours after CSA was administered, concentrations were low (64 ± 24 μg/L), with no inhibition of IL-2 production. A rapid, concentration-dependent response occurred. Maximum CSA concentrations (944 ± 187 μg/L) and maximum inhibition of IL-2 production (86.9% ± 13.7%) occurred 90 min after subjects received CSA. In vitro, 32.5–1200 μg/L CSA also inhibited PHA-stimulated IL-2 production in whole blood in a dose-dependent fashion with a similar IC₅₀ (~300–400 μg/L) ex vivo and in vitro.

Conclusion: In the search for a pharmacodynamic marker to better guide immunosuppressive therapy, the relationship between this simple, biologically relevant measure of CSA effect and clinical outcome should be determined.

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Nonstandard abbreviations: CSA, cyclosporine; IL-2, interleukin-2; IC₅₀, concentration producing 50% of the maximal inhibitory response; and PHA, phytohemagglutinin.
optimizing the clinical response to CSA to be focused on the optimization of drug concentrations by manipulation of dose to achieve target CSA concentrations within a poorly defined “therapeutic range” (11). However, it has long been noted that the clinical response to CSA, in terms of both efficacy and drug-related toxicity, may vary substantially even in patients with similar CSA concentrations (5, 12). Thus, interindividual pharmacodynamic differences in responses to CSA may be at least as important as pharmacokinetic differences in the determination of the overall clinical response to CSA.

The inhibitory effect of CSA on interleukin-2 (IL-2) production is central to its immunosuppressive action (13). Several investigators have attempted to use the inhibitory effect of CSA on IL-2 production or on IL-2-dependent lymphocyte proliferation as pharmacodynamic measures of response to CSA (14–19). Plasma concentrations of IL-2 in humans are low; thus, detection of a CSA-induced, concentration-dependent decrement of plasma IL-2 concentrations is difficult (14). Studies have, therefore, examined either the effects of plasma from patients receiving CSA on the proliferation of healthy lymphocytes from a different individual or have otherwise isolated lymphocytes from patients who have received CSA and examined the ability of these isolated lymphocytes in culture to proliferate or to produce IL-2 after mitogen stimulation.

These techniques have been problematic for several reasons. Plasma obtained from patients receiving CSA is not ideal for determining a pharmacodynamic response because ~50–70% of the drug concentrates in erythrocytes in a temperature-dependent fashion (5, 20). Thus, plasma CSA concentrations are much lower than whole blood CSA concentrations and are critically dependent on the temperature at which the plasma is separated from blood (5, 20). Plasma is therefore an unsuitable matrix for the measurement of both the pharmacokinetic and pharmacodynamic characteristics of CSA. The alternative strategy of using isolated lymphocytes in culture ex vivo to measure a pharmacodynamic effect of CSA is also flawed for several reasons. First, the isolation of lymphocytes from blood separates these lymphocytes from the complex, CSA-rich milieu in which their pharmacodynamic response occurs, and this may alter both response and rate of recovery (21). Second, isolation of lymphocytes involves multiple washes, which are likely to variably deplete the concentrations of CSA within the lymphocytes isolated. Third, the requirement for cell culture for 48 h or longer limits the practical application of any pharmacodynamic measure obtained using such techniques. Furthermore, because a concentration producing 50% of the maximal inhibitory response (IC50) for CSA (~3 µg/L) obtained using such techniques (19) is one or two orders of magnitude lower than typical CSA concentrations required in patients to prevent graft rejection, the clinical significance of such techniques is uncertain.

The lack of a biologically relevant, practical pharmacodynamic measure of the effect of CSA has been a serious obstacle to defining interindividual variability in response to CSA. The development of such a measure has remained an elusive goal (4, 5). We describe a practical pharmacodynamic measure of the effect of CSA determined in whole blood, both in vitro and ex vivo.

Materials and Methods

Materials

CSA and phytohemagglutinin-P (PHA) were obtained from Sigma Chemicals. Human IL-2 immunoassay kits were obtained from R&D Systems. CSA (1 mmol/L) was dissolved in dimethyl sulfoxide and subsequently diluted in sterile normal saline to a final working concentration of 10 µmol/L immediately before use. PHA was diluted to a concentration of 1 g/L in sterile normal saline and frozen in aliquots that were thawed immediately before use.

Experimental Protocol

All subjects provided written informed consent, and the study protocol was approved by the Vanderbilt Committee for the Protection of Human Subjects. Subjects were healthy, normotensive, non-smoking, male volunteers. No subject had clinically significant abnormalities on history, physical examination, or routine laboratory tests, including complete blood count and renal and liver function tests. Subjects did not take any medications for at least 2 weeks before each study day and abstained from caffeine and alcohol for 5 days before each study day. Subjects fasted overnight before each study day. All experiments were performed in the Vanderbilt Clinical Research Center with the subjects resting supine in bed. An intravenous cannula was placed in an antecubital vein. Thirty minutes later, baseline venous blood samples were drawn, and 4 mg/kg CSA was administered by mouth with 150 mL of water. Subjects remained fasting for 5 h after the administration of drug and were then fed a standardized lunch. Venous blood (5 mL) was drawn in an EDTA tube for determination of CSA concentrations and in a heparin-containing tube for measurement of PHA-stimulated IL-2 concentrations at the intervals indicated in each substudy.

Ex Vivo Studies

Study 1. To determine the relationship between the IL-2 inhibitory response and peak and trough CSA concentrations, venous blood was drawn from 18 subjects before and 4 and 24 h after the administration of 4 mg/kg CSA (Neoral®, Novartis). CSA concentrations and PHA-stimulated IL-2 production were measured as described below. We and others have described the methods for the measurement of cytokine production in whole blood (22–25). Duplicate 1-mL (total volume) samples containing 950 µL of whole blood and 50 µL of 1 g/L PHA (stimulated) or 50 µL of saline (unstimulated) were incubated for 6 h in a shaking water bath at 37 °C. At the end of the incubation, the samples were centrifuged in an Eppendorf microcen-
trifuge for 2 min, and the supernatant was removed and stored at −70 °C until assayed. Others have described a relatively narrow range of intraassay and interassay coefficients of variation (CVs; range, 6–18%) for stimulated cytokine production in whole blood (23). Because the CV in our initial studies, which used duplicate incubations, was small (11%), in subsequent studies single-sample incubations were performed. PHA-stimulated IL-2 production determined in blood obtained from each subject immediately before the administration of CSA represented the baseline (0% inhibition) response in that individual. Subsequent IL-2 production in samples obtained after CSA administration was expressed relative to the baseline IL-2 production.

Study 2. After study 1 (above) showed a rapid onset within 4 h and complete recovery of IL-2 inhibition within 24 h after the administration of CSA, we further defined the rapidity of onset of the response and its relationship to CSA concentrations as follows. Venous blood was sampled from five subjects before and 30, 60, 90, 120, 180, and 240 min after the administration of 4 mg/kg CSA (Sandimmune®, Novartis), and CSA concentrations and PHA-stimulated IL-2 production were measured as described. The Sandimmune formulation of CSA was used because it produces lower blood CSA concentrations than the Neoral preparation and would thus, by combining the data from studies 1 and 2, allow examination of the effects of CSA over a broader range of CSA concentrations.

IN VITRO STUDY

Study 3. Venous blood was drawn from four healthy volunteers into heparin-containing tubes, maintained at 37 °C, and used within 60 min. To measure the inhibition of IL-2 production by CSA in vitro, 50 μg of PHA and CSA in final concentrations of 0–1 μmol/L (0–1200 μg/L) were incubated with whole blood for 6 h in a total volume of 1 mL (850 μL of blood, 50 μL of 1 g/L PHA, and 100 μL of CSA) in a shaking water bath 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 −70 °C until assayed.

ASSAYS

IL-2 concentrations were measured by ELISA (R&D Systems). The lower limit of IL-2 detection was 31.2 ng/L. CSA concentrations in whole blood were measured using fluorescence polarization immunoassay (Abbott Diagnostics).

DATA ANALYSIS

Data are expressed throughout as mean ± SD. The decrease in PHA-stimulated IL-2 production in whole blood obtained from subjects after administration of CSA and in samples incubated with CSA in vitro was expressed as the percentage of inhibition relative to the baseline PHA-stimulated IL-2 response determined in the absence of CSA. Dose–response curves were fitted to a four-parameter logistic equation (Fig Perfect; Biosoft), and the IC50 for CSA was determined. Data that were gaussian distributed were analyzed by repeated measures analysis of variance and the t-test for paired or unpaired samples as appropriate. Data that were not gaussian distributed were compared using a Wilcoxon matched-pairs signed-rank test or a Mann–Whitney U-test as appropriate (SPSS for Windows, Release 6; SPSS). A two-tailed P value of <0.05 was the criterion for statistical significance.

EX VIVO STUDY 1: 4 AND 24 H AFTER CSA ADMINISTRATION

IL-2 concentrations in unstimulated whole blood incubated for 6 h were below the lower limit of detection of the assay, 32.1 ng/L. Ex vivo PHA stimulation of whole blood drawn at baseline, before subjects received CSA, produced a mean IL-2 concentration of 1790 ± 733 ng/L (n = 17). In the blood samples drawn 4 h after subjects had received CSA, the mean CSA concentration was 64 ± 196 μg/L and PHA-stimulated production of IL-2 was significantly decreased to 555 ± 345 ng/L (68.7% ± 17.2% inhibition; P < 0.0001; Fig. 1). For technical reasons, IL-2 production was not measured in two subjects at the 24-h time point. In the remaining 15 subjects, the mean CSA concentration was 64 ± 24 μg/L and PHA-stimulated IL-2 production was 2027 ± 855 ng/L 24 h after CSA administration. The absolute PHA-stimulated IL-2 production (ng/L) at baseline (time 0) and 4 and 24 h after CSA administration is shown in Fig. 2. Stimulated IL-2 production 24 h after subjects had received CSA was not changed from baseline (Figs. 1 and 2).

Blood from one subject produced 15-fold more IL-2 after PHA stimulation (estimated IL-2 concentration 33 000 ng/L) than was observed in any of the other subjects, whose mean stimulated IL-2 production was

![Figure 1](https://i.imgur.com/123456.png)
1790 ± 733 ng/L. These data, although unexplained, are unlikely to be artifactual because on a separate occasion, the same subject also performed study 2, and again this subject showed a magnified response. The IL-2 concentrations after PHA stimulation in this subject were far beyond the demonstrated linear range of the assay (2000 ng/L); thus, although the IL-2 response was inhibited 49% 4 h after CSA, his data were not included in the analysis.

**EX Vivo Study 2: 30–240 min After CSA Administration**

The time course of the inhibition of IL-2 production by CSA and its relation to the blood concentrations of CSA 30–240 min after the administration of 4 mg/kg CSA is shown in Fig. 3. A rapid, concentration-dependent, inhibitory effect of CSA on stimulated IL-2 production was observed. Maximum CSA concentrations occurred 90 and 120 min after the administration of drug (944 ± 187 and 953 ± 187 μg/L, respectively). Similarly, maximum inhibition of IL-2 production was also observed 90 and 120 min after the administration of drug (87% ± 3.1% and 84% ± 4.5%, respectively; Fig. 3). The inhibitory effect of CSA on IL-2 production decreased as CSA concentrations fell. Four hours after the administration of drug, the mean CSA concentration was 446 ± 67 μg/L and the mean inhibition of IL-2 production was 53% ± 19.0%.

**Study 3. In Vitro CSA Concentration Response**

CSA (32.5–1200 μg/L) inhibited the production of IL-2 in vitro in a dose-dependent fashion in blood drawn from all four subjects (Fig. 4). In the four individuals, the CSA IC50s were 280, 233, 256, and 347 μg/L, respectively.

The blood concentrations of CSA studied in vitro were chosen to span the range of blood CSA concentrations achieved in vivo after the oral administration of CSA in studies 1 and 2. The relationship between blood CSA concentrations and IL-2 inhibition in samples collected at 4 h and 24 h (study 1) and samples collected at 240 min (study 2) with each time point represented as an individual point is shown in Fig. 5. Superimposed is the solid line representing the mean (± SD) in vitro IL-2 inhibition by CSA as a concentration-response curve (study 3). The sigmoid concentration-response relationship is evident in both the ex vivo and in vitro studies as is the close relationship between the CSA concentration-response curves determined in vitro and ex vivo.

In one subject, the in vitro CSA concentration-response relationship (study 3) was determined in blood drawn immediately before the administration of 4 mg/kg CSA for study 2. Fig. 6 shows the excellent relationship between the in vitro IL-2 inhibition by CSA (concentration-
response curve) in this subject and the ex vivo inhibition of IL-2 resulting from the various concentrations of CSA that occurred after oral administration of CSA in the same subject on the same day.

**Discussion**

This study describes a simple technique for measuring a pharmacodynamic response to CSA and demonstrates (a) that the inhibition of stimulated IL-2 production in whole blood by CSA, both in vitro and ex vivo, is related to the concentration of CSA; (b) that after the administration of oral CSA, the IL-2 inhibitory response has a rapid onset and declines rapidly as concentrations of CSA fall; and (c) that there is considerable interindividual variability in IL-2 inhibition in response to similar concentrations of CSA.

The immunosuppressive action of CSA is thought to be mediated largely through effects on lymphocytes. After intracellular binding to cyclophilin, the CSA-cyclophilin complex binds and inhibits the action of calcineurin, reducing nuclear translocation of the cytoplasmic subunit of the nuclear factor of activated T cells to the nuclear subunit leading to decreased T-cell receptor transcription of the IL-2 gene (13, 26). Inhibition of IL-2 production is central to the immunosuppressive effect of CSA (13) because the inhibitory effect of CSA on lymphocyte proliferation can be reversed by the addition of exogenous IL-2 (13). Further evidence supporting the clinical importance of inhibition of IL-2 production has been provided by recent studies showing that blocking the IL-2 receptor with monoclonal antibodies reduced the frequency of transplant rejection (27).

The inhibition of mitogen-stimulated IL-2 in isolated peripheral blood lymphocytes by CSA has been studied as a potential pharmacodynamic measure of the effect of CSA (18, 28, 29). Such studies have generally shown that IL-2 production is inhibited by 35–40% in patients receiving CSA (18), but the cumbersome technique, which required isolation of lymphocytes, culture with mitogen for 48 h, and measurement of IL-2 by bioassay, as well as the highly variable interindividual responses and the lack of a well-defined association with CSA concentrations achieved in vivo have limited the practical application of this technique. Nevertheless, the potential importance of using IL-2 inhibition as a marker of the effect of CSA was demonstrated by the observation that failure to inhibit IL-2 production was associated with an increased likelihood of organ rejection (16). These studies thus suggested that a more refined measure of CSA-induced inhibition of IL-2 production might define a biologically relevant drug effect that would allow development of a pharmacodynamic measure of interindividual variability in response to CSA.

Our studies offer several practical advantages and new insights. The use of whole blood for IL-2 stimulation assays not only simplifies previous techniques, but also allows the pharmacodynamic effect of the actual concentration of CSA present in the blood to be determined in a milieu in which the in vivo response occurs. This avoids the confounding effects introduced by the lymphocyte isolation procedures, which not only remove the lymphocytes from the CSA-rich environment, but are likely to variably deplete intracellular concentrations of CSA. The whole blood technique thus allows the measurement of both CSA concentration and CSA effect in the same matrix, an ideal situation for defining the relationship between blood CSA concentrations and IL-2 inhibition ex vivo in 4- and 24-h post-CSA samples (study 1) and 30–240 min post-CSA samples (study 2). 

- and dashed line, results represented as individual points. Superimposed (solid line) is the concentration-response curve for in vitro IL-2 inhibition by CSA from study 3. ▲, mean values from study 3 (n = 4); bars, SD.

Fig. 6. In vitro (●) IL-2 inhibition by CSA (concentration-response curve) in one subject and ex vivo (+) inhibition of IL-2 in blood from the same subject on the same day at various concentrations of CSA after oral administration of 4 mg/kg CSA.
between concentration and effect. The 6-h incubation obviates the requirement for cell culture facilities, and measurement of IL-2 concentrations by ELISA further simplifies the technique.

Study 1 examined the effects of CSA 4 and 24 h after drug administration. These time points were chosen to approximately coincide with peak and trough drug concentrations and to provide initial information regarding the onset and duration of IL-2 inhibition. In a previous study that used isolated lymphocytes, the onset of action of a pharmacodynamic response was thought to be delayed 4–12 h after administration of CSA (18). In contrast, we demonstrated that a marked effect on IL-2 inhibition was present 4 h after CSA had been administered to subjects and that this effect had completely reversed 24 h after drug administration, although at that time CSA concentrations were 64 \( \pm \) 24 \( \mu g/L \), a concentration of CSA that, based on in vitro studies performed in lymphocytes, would be expected to have biological effects (15, 19). Our findings are similar to those of studies that measured the inhibition of calcineurin phosphatase activity by CSA and found that the effects of CSA are rapid in onset and are rapidly reversible (21).

Study 2 further characterized the onset of inhibition of IL-2 production by CSA and its relation to drug concentrations. The onset of the effect of CSA occurred rapidly, with peak inhibition of IL-2 production, \(-85\%\), occurring 90–120 min after drug administration and coinciding with peak CSA concentrations, which were \(-950 \mu g/L\) (Fig. 3). The IL-2 inhibitory effect of CSA decreased rapidly, in concert with the concentration of CSA, so that 4 h after administration, when blood CSA concentrations had decreased to \(-450 \mu g/L\), the IL-2 response was inhibited only 53\%. A small increase in IL-2 production was noted in some subjects at low concentrations of CSA both in vitro and ex vivo (Figs. 2 and 3). The mechanism underlying this observation is unclear, but it previously has been noted that in some patients IL-2 production in isolated lymphocytes was paradoxically increased 12–16 h after CSA, a time when low concentrations of drug are likely to have been present (18).

The in vitro incubation of whole blood with increasing concentrations of CSA produced a concentration-dependent inhibition of IL-2 production by CSA that was remarkably similar to the concentration-response relationship observed ex vivo in the blood of subjects who had received CSA. Thus from the fitted sigmoid dose-response curves, the estimated IC\textsubscript{50} concentrations of CSA in vitro (301 \( \mu g/L \)) and ex vivo (407 \( \mu g/L \)) were very similar. This finding contrasts with studies that used isolated lymphocyte techniques and found a median CSA IC\textsubscript{50} of 3–6 \( \mu g/L \) (15, 19) and is in agreement with studies that have used the inhibition of calcineurin activity (21) or the percentage of lymphocytes producing IL-2 (30, 31) as pharmacodynamic measures of CSA activity. The clinical relevance of our data is supported by the trough CSA concentration range, 100–400 \( \mu g/L \), empirically designated as therapeutic by various centers (7) and the observation that patients with steady-state CSA concentrations \(<350 \mu g/L\) during intravenous administration of the drug tended to have poorer graft survival (32).

The widespread clinical use of trough CSA concentrations to optimize therapy in patients has developed empirically. Thus, trough concentrations above a particular value, the absolute value of which varies two- to threefold according to the method used to measure CSA concentrations and the matrix (blood or serum) in which it is measured, have been associated with improved graft survival in some studies and have thus been designated a therapeutic target (4, 5, 7). The present studies suggest that IL-2 production is not inhibited at low concentrations of CSA and may not in fact be significantly inhibited during much of the dose cycle in some patients. The inability of trough CSA concentrations to reliably predict efficacy (6) may in part be explained by our observation that the immunosuppressive activity of CSA at trough concentrations may be low, thus rendering a measurement of trough CSA concentration a poor predictor of overall efficacy. Similarly, other studies that have attempted to improve the ability to predict efficacy on the basis of pharmacokinetic measures have used a composite measure of CSA disposition such as the area under the concentration–time curve or mean CSA concentrations. Our studies suggest that such composite measures of CSA disposition, which potentially include many time points at which CSA concentrations may not be associated with a significant immunosuppressive effect and time points at which CSA concentrations are in excess of those required for maximum effect, are unlikely to adequately predict immunosuppressive efficacy. Furthermore, our data show a scatter of pharmacodynamic responsiveness among individuals; therefore, similar concentrations of CSA produced a range of inhibition of IL-2. For example, a CSA concentration of 500 \( \mu g/L \) was associated with a 25–75\% inhibition of IL-2 production (Fig. 5). This interindividual variability in response to CSA is another explanation for the limited ability of pharmacokinetic measures to predict immunosuppressive efficacy.

We have shown that the CSA concentration-response curve is steep and that concentrations \(<150 \mu g/L\) have little effect, whereas those \(>900 \mu g/L\) produce little additional response. These findings suggest that it may be possible to define a therapeutic range of CSA concentrations based on this pharmacodynamic measure of response. Wide fluctuations in CSA concentrations may be counterproductive, producing little or no increased efficacy at concentrations above a certain threshold and limited efficacy at concentrations below a certain threshold. Thus, formulations of CSA that produce a pharmacokinetic profile with minimal peak-to-trough fluctuations in drug concentration may allow more effective immunosuppression.

There are several limitations to the present study. First, the use of a mitogen to stimulate IL-2 production is a
nonphysiological stimulus. Second, although inhibition of IL-2 is key to the effect of CSA, the drug has other effects relevant to its immunosuppressive activity. Third, the utility of the test in the setting of transplantation remains to be determined in future studies. Such studies would determine how the ex vivo lymphocyte IL-2 response may be altered by drugs other than CSA, by infection, and by rejection and would define the optimum pharmacodynamic target. In the transplant situation, each individual patient’s baseline lymphocyte IL-2 production could be determined before transplantation and thus provide a reference value against which CSA IL-2 inhibition, determined ex vivo while the patient is receiving CSA, could be targeted. Alternatively, it may turn out that an absolute degree of IL-2 inhibition is critical to optimal immunosuppression. If this is the case, then baseline values for individual patients will not be required. The degree of IL-2 inhibition that is important and how this should be optimally maintained over 24 h, in the critical posttransplant period, will also require study. Thus, studies that define the duration and degree of IL-2 inhibition required for optimum clinical efficacy will be of interest, as will be studies which examine the effects of chronic CSA dosing and the use of CSA in combination with other immunosuppressive drugs.

In conclusion, we have characterized a simple technique that provides a biologically relevant measure of the effect of CSA in vivo. The ability to measure response to CSA would allow immunosuppression to be optimized in individual patients. In the search for a pharmacodynamic marker to better guide immunosuppressive therapy, the relationship between this simple, biologically relevant measure of CSA effect and clinical outcome should be determined.

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References


