Advances in Cyclosporine Pharmacology, Measurement, and Therapeutic Monitoring

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This review will focus on our current state of understanding of the cellular and molecular mechanisms by which CsA exerts immunosuppression and will present new information on: pharmacokinetic variability of CsA, selection of specimen and method for measuring CsA concentration, identity of CsA metabolites and their immunosuppressive and toxic activity, and the relationship between steady-state pre-dose concentrations of CsA in blood and response to treatment in transplant patients.

In 1970 Sandoz scientists, in the continuing search for new and better medicines, retrieved soil specimens from the high plains area of southern Norway, Hardangervidda. Cyclosporine (CsA) was subsequently isolated from a new fungus strain, Tolypocladium inflatum Gams (1), obtained from those soil samples. Borel et al. discovered the immunosuppressive properties of CsA in rodent models used for the investigation of immunosuppression (2).

That fundamental discovery of what one investigator called an "immunoregulatory antibiotic" (3) has ushered in a new era of immunosuppressive therapy. CsA has had a major impact on our understanding and ability to selectively control the immune response. Since 1983 more than 6000 papers dealing with CsA have been published, with the current rate estimated at 1500 papers per year (4). The early promise of CsA as an agent that promotes the suppression of graft rejection has been realized in some significant ways. The overall improvement in graft survival for renal (5), heart (6), and liver transplants (7); the reduction in the number of life-threatening infections (8, 9); and the reported shortening of hospital stay (8) are benefits attributed to the use of CsA. Current immunosuppressive drug regimens used in many transplant centers are based on "triple drug therapy"—CsA, azathioprine, and prednisone. Some centers have maintained the strategy of using CsA and prednisone and, where possible, eliminating steroid from the regimen. The goal of eliminating steroid from the regimen in patients for at least one year and as long as five years has been achieved in low-responder living-related-donor kidney graft recipients with apparent success (10, 11). It is no surprise that the number of solid-organ transplant procedures performed annually has doubled since the introduction of CsA into clinical practice in December 1983. An extensive set of clinical trials are under way in attempts to take advantage of CsA's ability to control various autoimmune disorders (for a review, see reference 12).

These developments have presented scientists across a range of disciplines with challenges and opportunities to gain greater insight into key issues within their particular field of study. In the field of immunology, CsA is being used as a probe for understanding the mechanisms by which the immune system rejects foreign tissue at the cellular and molecular levels of organization. CsA has stimulated the investigation of immunopharmacologic control of autoimmune disorders and the study of mechanisms by which it exerts antiparasitic activity. CsA has challenged clinical chemists, clinical pharmacologists, transplant surgeons, and physicians, as perhaps no other single agent before, to develop the database that will improve our ability to optimize dosing of each individual patient. For clinical chemists and clinical toxicologists, responsibility for monitoring CsA therapy provides a major opportunity to investigate challenging problems as well as offer significant input to the field of organ transplantation. The recent report of the NACB/AACC task force on CsA monitoring extensively described critical issues that had arisen on this topic (13). Although the latter will be referred to in appropriate places, this review will focus on (a) our current state of understanding of the cellular and molecular mechanisms by which CsA exerts immunosuppression and (b) new information on the following topics: pharmacokinetic variability of CsA; selection of specimen and method for measuring CsA concentration; identity of CsA metabolites and their immunosuppressive and toxic activity; and the relationship between steady-state pre-dose concentrations of CsA in blood and response in transplant patients. A rigorous appreciation and application of the new knowledge gained in each of these areas can help further improve the chance of both short- and long-term success of transplantation in each patient.

Cellular Growth Regulation Mediated by CsA

CsA exerts a spectrum of cellular actions and striking effects on the growth of several different types of cells: it:
- inhibits helper and cytotoxic T cell activation and proliferation
- displays antiparasitic activity
- reverses resistance to chemotherapeutic agents
- enhances IgE production and T-helper cell priming in antibody and in delayed hypersensitivity responses
- reduces or eliminates skin lesions in psoriasis patients

Whether a similar molecular mechanism is at work in each of these systems is not known. The first studies that documented the immunosuppressive effects of CsA demonstrated a reduction of the immune response, primarily in mice, in the following tests: hemagglutinin production, formation of antibody-producing cells, rejection of skin grafts, experimental allergic encephalomyelitis, Freund's adjuvant arthritis, and graft-vs-host disease after transplantation of allogeneic spleen cells (14). CsA did not significantly depress leukocyte or thrombocyte counts in mice. Further studies demonstrated that CsA exerts its primary action on T, not B, lymphocytes (15). Thus, instead of suppressing the growth of all rapidly dividing lymphoid and myeloid cells, CsA exerts its primary effect on the immune response in a more selective way than do cytotoxic drugs such as cyclophosphamide and azathioprine (16).

CsA inhibits the lymphocyte-primed immune response during the first steps of the activation of T helper cells. However, once activation has proceeded to the state at

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which IL2 receptors are expressed on T cells, CsA is unable to inhibit IL2-dependent activation. This is a CsA-resistant pathway. CsA inhibition of T-cell activation is reversible and concentration dependent.

CsA exerts significant effects on cellular growth, not only in T lymphocytes that are responding to mismatched transplanted tissue or in T cells involved in autoimmune disease but also in certain other cell types involved in other disorders. For instance, CsA exerts an antiparasitic action against several but not all species of parasites (17). The resistance of cancer cell lines to specific forms of chemotherapy was reversed by CsA in Ehrlich ascites carcinoma in an animal model (18), in human T cell acute lymphocytic leukemia cells (19), and in a human small cell lung cancer cell line in vitro (20). The mechanism of this effect, as well as its possible clinical application, will undoubtedly be the subject of future investigations. A paradoxical effect of CsA is its stimulation of IgE production and T helper cell priming in response to standard antigenic challenges in several strains of mice (21). The possible mechanisms for this effect of CsA are described in reference 21.

Studies in psoriasis patients have shown that CsA administration eliminated psoriatic lesions partially or completely in most of the patients in an initial study (22-24). More extensive trials are under way. The cellular mechanisms that lead to psoriasis are a subject of vigorous debate (24). Does the hyperproliferation of epidermis result from a primary defect in keratinocyte growth regulation, or is it secondary to dermal influences (24)? Are T cells involved in the growth regulation abnormality? These are unanswered questions and, therefore, we do not yet know the cellular site(s) of action of CsA in this disease. But CsA definitely exerts a profound inhibition of the abnormal proliferation of cells that cause psoriasis.

**Mechanism of Inhibition of T-Cell Activation and Proliferation by CsA**

**Cellular Events**

The rejection of transplanted tissue and graft-vs-host disease are complex immune processes that involve the communication and interaction of a number of lymphocyte cell populations that, in turn, produce powerful and specific immunologic rejection mechanisms (25). The mixed lymphocyte reaction, in which cytotoxic T lymphocytes are generated and lymphokines are produced, is a widely used in vitro model of this process. In the mixed lymphocyte reaction, CsA inhibits early events in the activation and proliferation response of the recipient's lymphocytes to irradiated donor lymphocytes. The sites at which CsA exerts an inhibitory effect are thought to include the synthesis of IL2 by T helper cells and to a lesser extent, the synthesis of IL1 by monocytes. The production of these and probably other lymphokines, in addition to the direct interaction of various subsets of cells in the mixed lymphocyte reaction, leads to the expression of receptors for IL2 on the plasma membrane of cytotoxic T lymphocytes. Thus activated, the cytotoxic T lymphocytes undergo clonal expansion, when IL2 is present, and in turn directly attack donor tissue, causing cellular rejection. CsA inhibition of IL2 production suppresses the activation and subsequent proliferation of the cytotoxic T lymphocytes and T helper cell subsets of the T lymphocyte population. These and other inhibitory actions of CsA are concentration-dependent.

Thus, production of IL2, and possibly of other lymphokines and other gene products, is the lymphocyte function most sensitive to CsA. In the mixed lymphocyte reaction the usual concentration range of CsA that produces 50% inhibition of IL2 production is 10-20 μg/L (25). Typical CsA concentrations that produce 50% inhibition of the T-lymphocyte proliferative response in the mixed lymphocyte reaction are 20-50 μg/L (25). Inhibition of the formation of IL2 receptors or, alternatively, of their down-regulation, in precursor cytotoxic T lymphocytes in the mixed lymphocyte reaction is much less sensitive to CsA, the observed 50% inhibition being produced by CsA concentrations of 100-200 μg/L (25).

On the other hand, proliferation, in the presence of IL2, of T cells that already express the IL2 receptor on their plasma membrane, is resistant to CsA even at concentrations >1000 μg/L (25). Another population of T cells that are resistant to CsA inhibition are suppressor T cells (25). CsA's inhibition of T-helper-cell and cytotoxic T-cell activation and proliferation, while sparing inhibition of suppressor T-cell growth, is thought to be the key cellular event by which CsA prevents rejection and graft-vs-host disease and suppresses autoimmune disorders (25).

As important observations with possible implications at both the fundamental and clinical levels of our understanding of the mechanism of immunosuppression is the report of a pathway of T-cell activation, the CD28 pathway, that is associated with CsA-resistant expression of the IL2 gene (26). This pathway was not utilized by suppressor T cells, which are resistant to CsA as well (26), but was functional in T helper cells. The in vitro studies that demonstrated this CsA-resistant pathway used cultured peripheral blood lymphocytes obtained from the blood of healthy young adults. It will be important to learn if such a pathway is active in transplant patients who are experiencing acute rejection despite adequate concentrations of CsA in blood.

**Molecular Events**

**Effects on gene activation.** A multitude of genes are expressed in T lymphocytes when stimulated by certain mitogens (27-29). The stimulation of T cells by mitogens such as phytohemagglutinin or concanavalin A in vitro is generally regarded as a model for the receptor-mediated activation of T cells by foreign antigens in vivo such as those in mismatched transplanted tissue (30). Reed et al. (28) have recently shown that CsA, at a concentration of 1000 μg/L, suppressed the synthesis of messenger RNAs for IL2 by about 100%, for the proto-oncogenes c-myc and N-ras by about 80%, and for c-fos and IL2 receptors by about 50% in cultured human peripheral blood T lymphocytes stimulated by phytohemagglutinin. In this same study they also evaluated the effect of CsA on gene activation elicited by concanavalin A or IL2, in a cloned murine T cell line (25). The CsA specifically blocked the production of messenger RNAs for the c-myc and p53 proto-oncogenes when induced by concanavalin A but not when they were induced by IL2 (28). Apparently, more than one pathway controls the expression of a particular gene in T cells, and CsA selectively blocks only certain regulatory pathways of gene expression in these cells (28).

In another study, evidence was presented that more than 62 genes were activated in the early stages of T-cell activation by phytohemagglutinin and phorbol myristate acetate in the presence of cycloheximide (31). Using northern blots and run-on transcription techniques, investigators concluded that CsA inhibited the expression of about 50% of the activated genes, with no apparent effect on the
remaining activated genes (29, 31). On the other hand, when another type of cell, fibroblasts, are stimulated with serum, groups of genes similar to those in T cells are activated but CsA does not suppress their expression (31). The latter study demonstrates the tissue specificity of CsA's actions.

Mechanism of CsA inhibition of gene activation in T lymphocytes. A subject of great interest to investigators of the molecular aspects of the regulatory signals that govern T-cell gene activation is: which signals are inhibited by CsA and how is this accomplished? Although many biochemical events occur in T cells within minutes of antigen binding (32), the phosphatidylinositol pathway of "second messenger" production appears to play a central role in T-cell activation.

Briefly, when antigen interacts with T-cell receptor (or when mitogens such as phytohemagglutinin or concanavalin A interact with T-cell plasma-membrane receptors), the following cascade of molecular events is thought to occur: phospholipase C, in the endoplasmic reticulum, is activated, which in turn increases phosphoinositid turnover to produce diacylglycerol and inositol trisphosphate; the latter stimulates the release of bound calcium in the endoplasmic reticulum to produce an increase in the concentration of cytosolic free calcium; the increased free calcium activates calmodulin, which, together with diacyl glycerol, activates protein kinase c.

In vitro studies with phorbol esters (which bind to and directly activate protein kinase c) and calcium ionophores in place of antigens or mitogenic lectins have led to a two-signal model for T-cell activation wherein the combination of increased free Ca**+ and activation of protein kinase c appear to be required for the full activation of T cells (33, 34). Most reports indicate that CsA blocks events (including gene expression) induced by calcium ionophores but not those stimulated by phorbol esters (25). These and other studies indicate that CsA interferes with only some of the pathways that stimulate gene expression in T cells.

In considering how CsA inhibits T-cell activation, additional information at the molecular level needs to be taken into account. One or more cytosolic proteins bind CsA with reasonably high avidity. The best-characterized of these is cyclophilin, which is widely distributed in cells and tissues of eukaryotic organisms, has an Mr of 17 737, and is a basic protein with a high and relatively stereospecific binding avidity for CsA (Ka ~3 x 10**8 M/L) (35). Analyses of the amino acid sequence of cyclophilins demonstrated two regions that have homology to consensus sequences for ATP binding (35). One sequence is common to protein kinases and the other is common to ATPases. Harding and Handschumacher (35) have described the experimental conditions under which cyclophilin exhibits protein kinase activity and proposed that this catalytic function may be involved in gene activation in T cells. Quesniaux et al. (36) have shown that the interaction of CsA with cyclophilin is stereospecific and that the degree of binding of several CsA analogs is roughly parallel to their respective in vitro immunosuppressive activities. Other cytosolic proteins (Mr up to 200 000) that bind CsA have been described (36, 37), although thus far cyclophilin is quantitatively much more significant. If cyclophilin is involved in the activation of genes in T cells, one possible way CsA could block activation would be via a direct inhibitory effect on the putative protein kinase activity of cyclophilin.

Another hypothesis (32) is that cyclophilin provides a "sink" that makes it possible to achieve sufficiently high concentrations of CsA within cells to effectively inhibit activation at some as-yet-to-be-defined intracellular site(s). One such intracellular site is activated calmodulin. Thus, another suggested molecular mechanism by which CsA could inhibit T-cell activation is the inhibition of calmodulin activation through one of the two triggering pathways described above (25). Other investigators (36) have discounted this hypothesis because of not being able to demonstrate in vitro binding to calmodulin, whereas such binding has been demonstrated for cyclophilin in numerous laboratories. Possibly CsA causes inhibition of activated calmodulin by an as-yet-undiscovered indirect process.

A third possibility is that CsA interacts with putative T-cell-specific transcription factors, such as that which activates the IL2 gene (38), in such a way that the expression of certain genes is inhibited during the activation stage of T cells in the MLR (29, 38). What the putative T-cell-specific transcription factors are, and how CsA could modulate them, are subjects for future investigation.

A fourth possible mechanism is the inhibition of peptidyl-prolyl cis-trans isomerase, an enzyme recently shown (39, 40) to be identical in structure to cyclophilin. CsA inhibits its catalytic activity for the cis-trans isomerization of proline imidic peptide bonds, a reaction thought to be essential for protein folding during protein synthesis in the cell. Fischer et al. (40) postulate that in T cells this catalytic activity is necessary for the production of key proteins, in their fully active conformations, required for T-cell activation. Clearly much work remains to be done to elucidate the intricate mechanisms responsible for T-cell activation and how CsA inhibits that process.

Pharmacokinetic Variability of CsA

The extensive inter- and intrapatient variations in steady-state trough CsA concentrations in blood for a given

<table>
<thead>
<tr>
<th>Table 1. Factors Altering CsA Concentration in Blood*</th>
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<td><strong>Factors</strong></td>
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<tr>
<td>Absorption</td>
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<tr>
<td>Diarrhea</td>
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<tr>
<td>Liver disease (oral CsA)</td>
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<tr>
<td>Cystic fibrosis (oral CsA)</td>
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<tr>
<td>T-tube clamping</td>
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<tr>
<td>Bile salts</td>
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<tr>
<td>Metoclopramide (oral CsA)</td>
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<tr>
<td>Food</td>
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<td>Vehicle (milk, juice)</td>
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Elimination

| **Age**                                             | Decrease                      |
| Pediatric-increase clearance                        | Increase                      |
| Liver disease (intravenous CsA)                     | Increase                      |
| Other drugs                                         | Increase                      |
| Erythromycin                                        | Increase                      |
| Phenytoin                                           | Decrease                      |
| Phenoanbital                                        | Decrease                      |
| Carbamazepine                                       | Decrease                      |
| Ketoconazole                                        | Increase                      |
| Verapamil                                           | Increase                      |
| Cimetidine                                          | No change                     |
| Hemodialysis                                        | No change                     |
| Renal failure                                       | No change                     |

* Adapted from reference 13.
weight-adjusted dose of the drug are well known (13, 41). The extent and causes of variability in the absorption, distribution, metabolism, and elimination of CsA in transplant patients of all types were recently reviewed in detail in the report of the NACB/AACC task force on CsA monitoring (13). These will not be discussed again here, but the causes of the clinically most important changes in blood concentrations of CsA at trough are summarized in Table 1.

A valuable recommendation that provides the ability to individualize an optimal dosage of CsA on the first day of therapy is performance of pharmacokinetic studies before transplant surgery (42, 43). By performing pre-treatment oral and intravenous pharmacokinetic studies of the parent drug CsA over consecutive 24-h periods, possible problems such as poor absorption and (or) rapid or slow clearance can be identified and the initial doses adjusted accordingly. This approach provides the most accurate way to reach target blood concentrations in the shortest possible time.

In a recent evaluation of pre-transplant pharmacokinetics in heart–lung transplant candidates with cystic fibrosis (44), we used a mononclonal specific 3H-RIA method to identify poor absorption (ranging from 7% to 17%) in each of the six patients evaluated. In this study we uncovered a sub-population of patients with a pre-disposition to poor absorption, whose initial oral dosing in conjunction with transplant surgery must be increased accordingly.

In a recent study of the effect of time on CsA pharmacokinetics, the dose-adjusted trough concentration of CsA (HPLC method) in blood of renal-transplant patients at three months post-transplant was almost double the value obtained after one week (0.23 ± 0.13 vs 0.12 ± 0.06 ng/mL per milligram of CsA dose) (45). Further study will be required to determine whether increasing bioavailability of CsA or increasing hematocrit and plasma lipids and proteins produce the increase in dose-adjusted trough concentrations of CsA (45).

The circadian influence on CsA pharmacokinetics can be significant and is another source of intra- and interpatient variation (46). Eighteen of 24 paired am/pm 12-h trough concentrations of CsA (determined by HPLC) were lower by an average of 44.7% after a morning CsA dose than the average trough concentrations after an evening dose (46). These data emphasize the importance of measuring trough concentrations at a consistent time of day. The authors suggest that, in patients in whom the magnitude of these differences is large, optimizing immunosuppressive therapy with CsA might require lowering the evening dose, or raising the morning dose, or both (46, 47).

The extensive variation in CsA concentration in blood has led to the widespread monitoring of concentrations in blood (serum or plasma), to guide individualized dosing of the drug. A recommended schedule for monitoring CsA concentrations in renal-, heart-, and liver-transplant patients during the initial hospitalization is to measure at least every other day (13). Patients likely to have more variation in absorption or clearance in the early post-transplant period—e.g., recipients of liver and heart transplants, and patients with disease conditions or changes in concomitant therapy such as those noted in Table 1—will require more frequent monitoring to guide CsA dosing. Experience thus far has convinced most transplant centers of the need for periodic checks of CsA concentrations after the patient leaves the hospital as a measure of compliance and because of the potential for clinically significant drug interactions if concomitant drug therapy is altered (13).

### Selection of Specimen and Method for Measuring CsA

In considering the usefulness of measuring CsA concentration as a guide to immunosuppressive therapy, several hotly debated issues have developed, especially (a) the selection of the specimen in which to measure CsA and (b) the method for measuring CsA concentration. Regarding CsA measurement, it is also important to consider what is known about the ability of CsA metabolites to produce immunosuppressive and (or) toxic effects that are clinically important.

#### Selection of Specimen

Detailed discussions of the available data regarding the pharmacological and methodological issues involved in considering whether to measure CsA in whole blood, plasma, or serum have already been presented (13, 48). Whole blood has been recommended as the specimen of choice because the temperature- and time-dependence of the distribution of CsA between plasma (or serum) and blood cells can result in variations in plasma or serum concentrations that are avoided when whole blood is used. Furthermore, the use of whole blood precludes the problem of the increased relative amount of CsA in the plasma (or serum) compartment as the CsA concentration in the whole-blood sample increases (49). This in vitro concentration-dependent bias in plasma or serum concentrations of CsA, in addition to an in vitro bias produced by hematocrit changes, is most pronounced at lower temperatures (50). Much more constant ratios of whole blood to plasma CsA concentration are achieved at 37 °C. A third reason for recommending whole blood in preference to plasma or serum is that the higher concentrations of CsA in the former results in a higher signal-to-noise ratio in the drug measurement. This is all the more critical in view of the trend toward using lower dosages of CsA in transplant patients (48).

Thus far no compelling pharmacologic data have been presented that support the use of plasma or any of its fractions such as lipoproteins or plasma water as the recommended specimen for CsA measurement instead of whole blood. CsA appears to distribute rapidly and reversibly between blood cells and plasma proteins at 37 °C (48). In fact, recent studies have shown that the binding affinity of CsA-binding protein (cyclophilin) in erythrocytes decreases with increasing temperature, reaching negligible values at 42–43 °C (51). We have observed a rapid uptake of CsA from perfusion fluid (physiological concentrations of electrolytes and either albumin only or albumin plus rat blood) by isolated perfused rat kidneys at 37 °C. Tissue concentrations much higher than that in the circulating perfusate are reached in this model system (K. Roby and L. Shaw, unpublished observations). Human kidney concentrations of CsA and metabolites are several-fold higher than the corresponding whole-blood concentrations (13) in renal-transplant patients. These observations have led me to be a proponent of the hypothesis that tissue-binding affinity for CsA is greater than that of any blood constituent at physiological temperature and is the most important factor accounting for CsA distribution between binding sites in tissues and in blood constituents. If this hypothesis is correct, CsA concentration in blood is an accurate measure of the concentration of drug that is presented to tissues.
Method for Measuring CsA

During the final clinical trials of CsA therapy, two primary methods for monitoring the drug's concentration were introduced. One was the 3H-RIA developed and manufactured by Sandoz, Ltd. (52), a procedure originally based on the use of rabbit polyclonal CsA antibodies, which were later replaced by sheep polyclonal CsA antibodies. Both antibodies exhibited cross-reactivity toward several CsA metabolites in addition to reacting with the parent drug. The other method, based on high-performance liquid chromatography (HPLC), specifically measures the parent drug (48). Many studies in which any one of these methods was used to measure CsA or CsA plus metabolites in either serum, plasma, or whole blood have been described in the literature (13). These early reports provided some basic understanding about the use of CsA measurements in attempting to individualize the CsA dose. The use of virtually all possible combinations of methods and sample matrices in these studies produced a bewildering array of concentrations and made inter-center comparison of CsA monitoring a difficult, if not impossible, task. In the same sample matrix, nonspecific methods (i.e., those that measure parent drug plus metabolites) can produce concentration values as much as 51-fold higher than the respective concentrations of the parent drug (53).

The recommendation to measure the parent drug in whole blood by specific HPLC or monoclonal immunoassay methods (13) is based not only on the value of making studies from different centers more readily interpretable but also on (a) the fact that the parent drug possesses potent concentration-dependent immunosuppressive activity and nephrotoxicity and (b) the value of knowing what is being measured. As discussed below, the immunosuppressive activity of CsA metabolites is a hotly debated question, although most investigators agree that some metabolites, especially metabolite 17, possess at least a small degree of immunosuppressive activity. The few published studies of metabolite nephrotoxicity indicate little if any toxicity, as compared with the parent compound. When using a non-specific immunoassay alone, it is virtually impossible to know how much parent drug and what concentrations of individual CsA metabolites are reported in each measurement.

Further compounding this problem is the observation that the degree of cross-reactivity of three different polyclonal immunoassays towards specific CsA metabolites varies considerably (Table 2), emphasizing the point that different polyclonal antibody preparations will give concentration values that reflect very different proportions of CsA metabolites and the parent drug (64). Thus each polyclonal immunoassay measures something different, and any suggestion that they are comparable with each other or with specific methods is erroneous.

Any present or future attempts relating parent drug concentration and possibly concentrations of one or more specific metabolites to short- and long-term post-transplant clinical events will be difficult, if not impossible, with the current nonspecific measurements. Supplementation of parent drug measurement with an evaluation of changes of the combined CsA plus metabolite concentrations in relation to clinical events, with use of a nonspecific monoclonal antibody 3H-RIA procedure, may be a useful tool in an initial search for clinically important metabolite changes.

Sandoz scientists developed a monoclonal antibody (55–57) that showed little cross-reactivity toward 11 CsA metabolites (57). Using this antibody, Sandoz has developed a 3H-RIA specific for CsA. Incstar Corporation has also produced an RIA kit, using the same monoclonal antibody but with an iodinated CsA ligand that provides concentrations for whole-blood samples comparable with HPLC-measured values (58). E.I. du Pont de Nemours and Co. Inc. and Syva Co. are also developing immunoassays with the Sandoz specific monoclonal antibody. These developments will make possible the widespread use of specific measurements of the parent drug CsA in therapeutic monitoring and transplantation programs throughout the world. Indeed, the recent report of the United Kingdom CsA Quality Assessment Scheme (59) noted a trend toward the use of specific methods for CsA measurement. In July 1987, 26% of reported results were for specific methods; by August 1988, the percentage had risen to 57% (59). Although these developments suggest that interlaboratory comparisons of CsA monitoring data will be very much facilitated in the near future, a note of caution is appropriate: initial results from the UK Quality Assessment Scheme indicate that further refinements in methodology will be required to improve the analytical performance of the specific methods (59, 60).

CsA Nephrotoxicity

Nephrotoxicity is generally considered to be the clinically most important adverse effect of CsA (61). Dose-response studies in rat models have shown that CsA nephrotoxicity is expressed acutely as a dose-dependent increase in renal vascular resistance, a decrease in renal blood flow, and a decrease in glomerular filtration rate (62). These early changes are functional, fully reversible when the drug is withdrawn, and involve no major permanent structural changes (63, 64). When administered chronically to rats in high doses, 25 mg/kg intraperitoneally daily for 28 consecutive days, significant functional and structural changes can be produced (65). The glomerular filtration rate decreased to 30% of the control value and there was a consistent appearance of several structural changes, including patchy interstitial fibrosis, tubular atrophy, interstitial inflammation, and marked hypertrophy and hyperplasia of the juxtaglomerular apparatus (65). The CsA concentration in blood 24 h after the last CsA dose averaged 4712 µg/L, more than 20-fold higher than the usual trough concentrations obtained in transplant patients.

In humans, as in rats, it is generally agreed that the characteristic hemodynamic effects of CsA are an increased renal vascular resistance, a decreased renal blood flow, and a consequent decrease in glomerular filtration rate (66). There is also a consensus that these acute effects are decreased by reduction of CsA dosage. The nature of the vasoconstriction remains to be fully described (66). Curtis and Laskow summarized the two possible sources of the renal vasoconstriction (66): the role of vasoconstriction of afferent or efferent arterioles as the vascular cause of the

| Table 2. Cross-Reactivities of CsA Metabolites in Three Polyclonal Immunoassays* |
|----------------|---|---|---|---|---|---|---|---|
|               | CsA | M17 | M18 | M21 | M25 | M203-218 | M13 | M1 |
| Sandoz RIA    | 100 | 50  | 17  | 4   | <1  | 7         | 21  | 44 |
| Incstar RIA   | 100 | 60  | 79  | 14  | 8   | 20        | 13  | 41 |
| Abbott TDX FPIA | 100 | 116 | 57  | 54  | 52  | 51        | 26  | 14 |

* Lensmeyer et al. (54).
renal vasoconstriction, and (or) a direct effect of CsA on the glomerular capillary, which changes the ultrafiltration characteristics of this capillary system. Chronic CsA nephrotoxicity has been observed in some transplant patients (67). Minimizing acute nephrotoxicity by maintaining whole-blood concentrations of CsA within a narrow range of concentration reduces the chance of developing acute nephrotoxicity, which, in turn, should reduce the possible development of chronic nephrotoxicity (13, 53). Curtis and Laskow suggest that pharmacological studies aimed at reversing CsA-induced vasoconstriction will help to further elucidate the mechanism of nephrotoxicity and provide the means for controlling it clinically (68).

It is likely that certain factors, including endotoxin (68), renal ischemic injury (69), aminoglycoside antibiotics (70), other nephrotoxic agents such as amphotericin B, pre-existing kidney disease in non-renal-transplant patients, and possibly other unidentified influences, will predispose patients to the nephrotoxic effects of CsA. Patients whose kidneys are impaired by any of these factors may be at greater risk than others for developing nephrotoxicity, even when their CsA concentrations in whole blood are within the target range (68).

Immunosuppressive and Toxic Activity of CsA Metabolites

CsA is extensively metabolized by the liver cytochrome p450 complex. At least 19 metabolites—1, 8, 9, 10, 13, 16, 17, 18, 21, 25, 26, 203-218, H230, H235, H310, H315, H355, H410, and H420—have been detected in transplant patients' blood, bile, and urine with use of a gradient HPLC method (71). Although the structures of the first 12 of these have been elucidated in mass spectroscopy and nuclear magnetic resonance studies (72–74), the structures of the others remain to be determined. The structurally characterized metabolites are produced by metabolic reactions involving hydroxylation, demethylation, oxidation, and cyclization of CsA (Figure 1). HPLC methods have been developed for measuring CsA metabolites in biological fluids and tissues (75–80).

Considerable efforts have been made to determine whether any of the CsA metabolites are immunosuppressive. Several investigators, using in vitro tests of immunosuppression, have reported little or no immunosuppressive activity for metabolites (74, 81–83). Others have reported significant activity for a few metabolites, especially metabolite 17 (84–86), either by itself (84, 85) or synergistically with CsA in a clone of T lymphocytes cultured from heart-tissue biopsy obtained during a rejection episode (86).

The reason for the difference in results among these studies is not clear. One suggested way to settle this issue is to use an animal transplant model, which would avoid potential biases that might occur in in vitro model systems and to examine the ability of the metabolites, in comparison with CsA, to prevent rejection of the transplanted tissue. Now that sufficient quantities of some metabolites are being purified from human bile, it should be possible for some investigators to test for in vivo immunosuppressive activity of CsA metabolites. Indeed, Wong and collaborators, using rat small intestine as a transplant model, are comparing metabolite immunosuppressive activity with that of CsA (87). Studies such as these should presumably lead to an answer to the question: do any CsA metabolites exert a clinically significant immunosuppressive effect?

Are any CsA metabolites nephrotoxic? This question has been tested directly in two published studies thus far (88, 89). Ryffel et al. (88), in their spontaneously hypertensive rat model for CsA nephrotoxicity, evaluated the toxic potential of purified metabolite 17 and of a mixture of CsA metabolites extracted from transplant patients' bile. This group reported that, after four weeks of daily intraperitoneal administration of metabolite 17 or the biliary metabolite mixture, 10 mg/kg, neither functional nor morphologic changes were observed (88). In contrast, the same dosage regimen of CsA in the standard olive-oil vehicle produced a substantial decrease in glomerular filtration rate and morphologic alterations: exudative arteriopathy and regenerative renal tubular changes (88). The acknowledged limitation in these detailed direct studies of the toxicity of metabolite 17 is the lack of specific measurement of the metabolite in the blood or tissues of the rats.

Cole et al. (89) evaluated the effect of purified metabolite 17 on the growth of pig renal tubular cells, in comparison with the effects of CsA. Metabolite 17, up to 10 mg/L, exerted minimal effects on synthesis of DNA, RNA, or protein in all three cell types. In contrast, CsA at 500 μg/L inhibited protein synthesis by about 40% in the tubule cells and by about 60% in the mesangial cells, but only slightly in the non-renal cells (89). Investigations are in progress evaluating the possible toxicity of metabolite 17 and other purified metabolites in human renal tubule cells (L. Bowers, personal communication) and in the isolated perfused rat kidney (L. Shaw, unpublished).

Rosano et al. (90) reported the appearance of high concentrations of unidentified CsA metabolites more polar than metabolite 17 in blood from a renal-transplant patient at the onset of an episode of CsA-related nephrotoxicity. The questions raised by these observations are: is CsA-related nephrotoxicity always accompanied by an increase in concentrations of CsA metabolites? Which metabolites are involved? Are these metabolites a causative factor in CsA nephrotoxicity, or do they accumulate secondary to impaired renal clearance?

Several laboratories are investigating the possible correlation between CsA-related nephrotoxicity and the appearance of significantly increased concentrations of CsA metabolites in transplant patients. These clinical studies and those involving the models of immunosuppression and nephrotoxicity noted above will, it is hoped, help answer the questions: do we need to monitor CsA metabolites, in addition to the parent drug, to optimize immunosuppressive therapy and, if so, which ones?
Cyclosporine Monitoring

Because of the striking intra- and interpatient variability in the absorbance and clearance of CsA and its narrow therapeutic window, most centers have adopted regular monitoring of CsA concentrations in blood (serum or plasma) to guide dosage. The early experience regarding the utility of monitoring CsA concentrations in most studies was that there was a degree of correlation between the drug’s concentration and the occurrence of nephrotoxicity (CsA concentrations relatively high) or transplant rejection (CsA concentrations relatively low) (48). In several ongoing or published (9, 91, 92) studies, the dosage of CsA was adjusted to maintain whole-blood concentrations at trough within a narrow range to reduce the risks of rejection and nephrotoxicity.

In the CsA era of immunosuppressive therapy, patient and graft survival rates are higher than ever before. The goal of transplant centers is to further improve the clinical outcome for each transplant patient, with the ultimate goal being earlier detection and control of acute rejection and chronic nephrotoxicity. In my opinion, the regular adjustment of CsA dosage to achieve and maintain whole-blood concentrations within a narrow range at trough and possibly to allow a pharmacokinetic evaluation of each patient can contribute to a further improvement of clinical outcome. Other improvements such as the development of a simple and reliable method for the early detection of acute rejection could provide additional help.

Two recent studies document clinical outcomes for renal-transplant patients whose whole-blood concentrations of the parent drug, measured at trough by a specific method (HPLC), were maintained within narrow ranges. In the first of these (9) the clinical characteristics for 39 patients receiving CsA plus prednisone therapy, and followed for 15 to 18 months after transplant surgery, were as follows: 13 received kidneys from living relatives, 26 from cadaver donors; for 27 patients the kidney transplant was their first, and for 12 (31%) it was their second or third. The following clinical outcomes were obtained: 17 (44%) encountered at least one episode of acute rejection within the first year after transplant surgery, with 70% of these occurring within the first four months. Fourteen of these 17 patients had a whole-blood concentration of CsA at trough of <125 μg/L at the time of the episode; the remaining three (18%) had concentrations at the lower end of the target range. Only two of the 39 patients had episodes of CsA-related nephrotoxicity, and in both the whole-blood concentrations of CsA at trough had been at the high end or above the target ranges, 262 and 361 μg/L, at the time of the nephrotoxic episodes. Moyer et al. (9) made no mention of whether they detected either chronic rejection or chronic nephrotoxicity in these 39 patients. For the duration of the study, serial measurements of serum creatinine remained stable, with an average value of 17.9 mg/L at 15–18 months post-transplant. Iothalamate clearances were performed on the majority of these patients and showed no deterioration in the mean end-of-study value (46.6 mL/min per 1.73 m²) compared with an initial mean value of 41.9.

In the second study (91) whole-blood trough concentrations of CsA were also maintained within a narrow range. Whenever they were found to be outside the range, dosage was adjusted to bring the concentration back within the range, regardless of whether clinical events such as nephrotoxicity or rejection were occurring. In this study Uchida et al. (91) followed 127 adult renal-transplant recipients (76 living related donors, 51 cadaver donors) for up to four years. Concentrations of CsA in whole blood were measured by HPLC in specimens obtained just before the morning dose. Dosage adjustments were made to keep trough blood concentrations of CsA within 200 (±20) μg/L for the first three months after transplant surgery for the recipients of kidneys from a living relative. After three months, the dosage for these patients was reduced to achieve blood concentrations within 100 (±20) μg/L. For the recipients of cadaver kidneys the target trough blood concentration of CsA was 100 (±20) μg/L in the immediate post-transplant period as long as the serum creatinine values were ≥30 mg/L (91). If serum creatinine values dropped to <30 mg/L, the CsA dosage was adjusted to achieve 200 (±20) μg/L trough blood concentrations for the first three months post-transplant, then reduced to maintain trough concentrations within 100 (±20) μg/L. These concentrations were determined three times per week during hospitalization and at every outpatient clinic visit thereafter. In this study, when serum creatinine concentrations increased by more than 25% above the baseline value with no evidence of rejection, the CsA dosage was decreased (91). If rejection was suspected, a graft biopsy was performed. When CsA concentrations were outside the target ranges in the absence of rejection or toxicity, the dosage was adjusted to bring them back within the appropriate range.

The clinical outcome for patients in this study was as follows. Acute rejection episodes occurred in 46 (36.2%) of the 127 patients: for the 37 patients who experienced acute rejection within the first 90 days post-transplant, CsA blood concentrations were within the target range; for the nine who experienced acute rejection after 90 post-transplant days, concentrations in blood were below the lower limit of the target range, 80 μg/L. A total of 17 patients (13.4%)—11 treated for acute rejection and six who had no acute rejection at all—developed chronic rejection. Five patients (3.9%) developed mild chronic renal dysfunction, presumed to be nephrotoxicity because there was no evidence of active rejection episodes on biopsy examination. There was no evidence in any of these patients of progressive deterioration of renal function. No indication was made in this report of the number of patients who had prior kidney transplants and who, therefore, would be at greater

| Table 3. Recommended Target Ranges for CsA Concentrations (μg/L) in Whole Blood |
|---------------------------------|---------------------------------|
| Transplant                     | First 3 to 6 mo. post-transplant | After 3 to 8 mo. post-transplant |
| Kidney                          |                                 |                                |
| Living-related donor            | 150–250                         | 80–125                         |
| Cadaver donor                   |                                 |                                |
| Serum creatinine ≥30 mg/L       | 80–120                          | 80–125                         |
| Serum creatinine <30 mg/L       | 150–250                         | 80–125                         |
| Liver                           |                                 |                                |
| Absence of toxicity             | 250–350                         | 100–150                        |
| Presence of toxicity            | 80–120                          | 100–150                        |
| Heart                           | 350–450                         | 100–150                        |
| (day 1–7)                       | (day 8–90)                      | (day 91–180)                   |
|                                | 250–350                         |                                |
|                                | (day 8–90)                      |                                |
|                                | 150–250                         |                                |

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risk for rejection.

Based on these studies, the target ranges summarized in Table 3 are recommended for monitoring the concentration of the parent drug CsA in whole-blood samples obtained at trough from renal-transplant patients. Further long-term prospective studies to confirm the proposed therapeutic drug monitoring guidelines are needed. Included in the final report of such a study should be the incidence of acute and chronic rejection, acute and chronic nephrotoxicity, and, of course, patient- and graft-survival data. The proposed lower concentration limit of 150 μg/L for minimizing renal-graft rejection within the first three months after surgery appears to be substantiated by the recent study of Holt et al. (92). These investigators, using CsA concentration values measured in two to six blood specimens collected 8–10 h after the last drug dose within the seven days preceding diagnosis, found that 14 of 32 rejection episodes were associated with median CsA concentrations of <200 μg/L. This cut-off value for the parent drug in whole-blood specimens collected 8–10 h after the last dose is roughly equivalent to 150 μg/L in specimens collected 11–12 h after the last dose (D. Holt, personal communication).

Investigations of the relationship between CsA concentrations and clinical events in heart, liver, and other transplant patient populations are under way. However, there are no definitive published studies on this subject. For liver-transplant patients Wonigeit (93) has discussed provisional guidelines for CsA whole-blood concentrations similar to those discussed above for renal-transplant patients: >200 μg/L in the first few months post-transplant and 100 to 200 μg/L thereafter. Should nephrotoxicity, neurotoxicity, or hypertension attributable to CsA develop in liver-transplant patients in the early post-operative period, the Mayo Clinic liver-transplant team recommends lower CsA dosage to achieve target whole-blood concentrations of CsA at trough of 80 to 120 μg/L and adding azathioprine to the immunosuppression regimens (94). The latter group otherwise uses a target CsA concentration range of 250 to 350 μg/L for the first few months after surgery, which is similar to that suggested by Wonigeit. A suggested set of target ranges for heart-transplant patients follows the same overall concept of lowering the blood concentrations upon stabilization of graft function (T. Schroeder, personal communication; see Table 3 for details).

Another aspect of monitoring CsA concentrations in whole blood, which merits consideration, is the use of pharmacokinetic studies to guide CsA dosage. Recent reports suggest that the use of pharmacokinetic evaluations of CsA in renal-transplant patients can provide more predictive information about the likelihood of subsequent rejection (95, 96). This area of CsA monitoring is deserving of further evaluation to establish the potential benefits vs monitoring trough concentration, in terms of the respective costs involved.

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References

Cyclosporin A-mediated inhibition of mitogen-induced gene transcription is dependent on cell type and mitogenic stimulus. (submitted)


