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COMMENTARY

T-LYMPHOCYTE INHIBITION BY CYCLOSPORINE

POTENTIAL MECHANISMS OF ACTION*

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Cyclosporine (CsA) is an eleven amino acid cyclic peptide derived from two strains of *Fungi imperfecti*. A potent immunosuppressive agent, CsA is unlike other agents in that it lacks significant myelotoxicity and appears to specifically inhibit T-lymphocyte-mediated immune responses.

Since the original description of the immunosuppressive effect of CsA by Borel *et al.* in 1976 [1], an enormous body of literature has accumulated concerning the *in vivo* and *in vitro* effects of this novel immunosuppressive agent. An extensive review is beyond the scope of this commentary. Analysis of the data, however, can be organized into three categories and conveniently summarized: *in vivo* laboratory animal and clinical studies; *in vitro* studies at the T-lymphocyte subset level; and *in vitro* studies at the subcellular level. The last section, a summary of those studies analyzing the effects of CsA at the subcellular level, will form the major emphasis of this commentary.

In a variety of animal models, CsA facilitates the induction of specific transplantation tolerance. This state of tolerance appears to be a process specific for the initial tolerance-producing antigen, since third party antigens in the form of subsequent transplants are promptly rejected [2, 3]. This state of unreactivity also appears to be a dynamic, true tolerance, since withdrawal of CsA does not appear to influence graft retention in a number of animal models [4]. In humans, CsA has been used primarily in solid organ transplantation, with immediate success. CsA combined with low dose steroid administration has become the immunosuppressive regimen of choice in most transplant centers. The immediate marked improvement in clinical cardiac and liver transplantation with CsA use has removed these procedures from the realm of experimental surgery to that of accepted procedures for end-stage cardiac and liver disease. Subsequently, the clinical use of CsA has been expanded to prevent graft-versus-host disease following bone marrow transplantation for leukemia. Investigational use of CsA has also begun for the treatment of a wide variety of presumed immune disorders, including rhematoid arthritis, primary pulmonary fibrosis, juvenile onset diabetes mellitus, retinitis pigmentosa, and a variety of hematologic disorders (aplastic anemia, primary red cell aplasia).

Despite this expanding, empiric clinical application of CsA, knowledge of the precise cellular and subcellular mechanisms of action of CsA is incomplete. Detailed information on the exact biochemical site of action of CsA in specific cells involved in the immune response is critical not only for the rational clinical use of this immunosuppressive agent but also to explain the toxicity of CsA on other organ systems, to design optimal clinical immunosuppressive regimens using combinations of agents acting at different points in the immune response, and to direct the development of CsA analogs or other agents that may provide specific immunosuppression with less toxicity. The demonstration of the exact mechanisms of action of CsA will also provide further understanding of the cellular and subcellular biochemical basis of the primary and secondary immune responses of T lymphocytes.

Initial investigations at the cellular level, using unmodified CsA, have focused primarily on the T lymphocyte and T-lymphocyte subsets, following the reports of its apparent selective action on T lymphocytes by Borel [5]. More recent studies, however, have demonstrated a CsA effect on both T-independent B cell and macrophage responses, as well as effects on other organ systems [6, 7]. From these largely descriptive investigations a number of useful facts concerning the mechanism of CsA can be derived. First, CsA has differential effects on naive T-lymphocyte subsets. The T-helper cell and its production of lymphokines, primarily interleukin-2, is the most sensitive subset [8]. The maturation and generation of the precursor cytotoxic T cell are also sensitive to the CsA effect [9]. The T-suppressor cell, however, is much more resistant to the effect of CsA [10]. The generation and maintenance of a population of specific suppressor cells uninhibited or facilitated by CsA may explain the ability of CsA to induce specific transplantation tolerance in experimental animal models. The second important finding in these initial series of experiments is the narrow time frame of the CsA effect, that is, activated lymphocytes are largely resistant to the effect of CsA. It appears that, once lymphocytes have been primed with antigen (48–72 hr), the addition of CsA has little or no effect on proliferation in response to rechallenge with antigen, while new primary responses to a second antigen are inhibited [11, 12].

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Thus, it appears that CsA interferes with an early step in T-lymphocyte activation following initial antigen presentation, to prevent both lymphokine production by the T-helper cell and the ability of the cytotoxic T lymphocyte to respond to lymphokines, while suppressor cell activation and generation are largely unaffected [13]. Similarly, CsA appears to influence the ability of subsets of monocytes to function in antigen presentation and interleukin-1 production [14]. The effects of CsA on other monocyte activities are currently being assessed.

Studies from a number of laboratories have examined the effect of CsA of T lymphocytes at the subcellular level. These studies have examined the effects of CsA on a variety of cell populations. A major limitation of many of these studies has been the use of malignant T-cell leukemia cell lines or activated lymphoblasts. Interpretation of the effects of CsA on the metabolic activities of these cell lines is difficult. Extrapolation of these data to the effect of CsA on the naive T lymphocyte, therefore, must be approached cautiously. A second limitation of many studies has been a lack of attention to the doses of CsA used, or failure to demonstrate appropriate dose-responsive curves.

These studies can be discussed within the framework of a proposed schema for T-lymphocyte activation proceeding from the cell membrane to the nucleus. Evidence is accumulating for a two-step activation sequence for T lymphocytes. The first step appears to be membrane receptor binding by mitogens or alloantigen in association with macrophage Class II antigen (HLA-DR), which creates membrane perturbation and a rise in intracellular calcium [15]. This secondary rise in intracellular calcium activates or facilitates the activities of a number of cell proteins or enzymes. These proteins include calmodulin and the related proteins, phospholipase A_2 and phospholipase C. Each of these proteins has been reported to have a central role in cell activation and to regulate many cell activities through various second messenger systems: cyclic nucleotides (calmodulin); arachidonic acid turnover—prostaglandins/leukotrienes (phospholipase A_2) and diacylglycerol/inositol triphosphate (phospholipase C) [16–18]. The relative importance of each of these calcium-dependent proteins for T-lymphocyte activation is currently under intensive investigation in a number of laboratories. Activation of another enzyme, protein kinase C, by diacylglycerol, phospholipids and calcium appears to be a second important step in T-lymphocyte activation. Lymphokines such as interleukin-1 and interleukin-2 may provide the necessary second signal for full T-cell activation through activation of protein kinase C. Phorbol esters, which appear to directly activate protein kinase C, may substitute for these lymphokines during T-lymphocyte activation [19].

Following the cytoplasmic activation of calmodulin-dependent protein kinases, cAMP-dependent protein kinases and protein kinase C, the T-lymphocyte activation sequence progresses to nuclear induction of ornithine decarboxylase and polyamine synthesis necessary for gene activation and expression, mRNA and DNA synthesis, and cell cycle progression to cell division [20].

As alluded to above, the initial clue to the subcellular mechanism of action of CsA is the narrow time constraint of its effect. The addition of CsA to an established T cell line does not inhibit proliferation in the presence of interleukin-2 [21]. Similarly, proliferation of T lymphocytes in response to mitogens or alloantigen is not inhibited by the addition of CsA 36 or 72 hr following stimulation respectively. Cell cycle analysis following mitogen stimulation of T lymphocytes demonstrates that CsA prevents cells from proceeding to S phase of the cell cycle, with accumulation of cells in late G_1 phase [22]. These studies indicate that CsA prevents signals for DNA synthesis/replication. Other investigations demonstrate that CsA inhibits the synthesis of mRNA and new protein synthesis required for cell proliferation [23, 24]. The induction of ornithine decarboxylase is inhibited by CsA as early as 4 hr following mitogen stimulation [25]. This enzyme is the rate-limiting step in the polyamine synthesis required for DNA and RNA synthesis. The production of mRNA for interleukin-2 appears to be inhibited by CsA, using T lymphoblasts and Northern Blotting techniques. Along similar lines, the mRNA for the c-myc oncogene product is likewise inhibited by CsA, whereas the mRNA for c-fos and the interleukin-2 receptor do not appear to be inhibited by CsA [26]. From these studies, it appears that CsA interferes with one or both of the early activation signals in the first few hours following mitogen or alloantigen stimulation of susceptible T lymphocytes, preventing activation of the genes necessary for the mRNA production that is required for the synthesis of certain oncogene products as well as interleukin-2.

In a number of studies, the effects of CsA on these signals have been investigated independently. The signal that is apparently linked to lymphokines and protein kinase C does not appear to be influenced by CsA since potentiation of mitogen responses (proliferation) by phorbol esters is not inhibited by CsA [27]. Similarly, proliferation of activated T lymphocytes in response to interleukin-2 is not inhibited by CsA. A major criticism of these studies is the assumption that the effect of the phorbol esters is mediated exclusively via activation of protein kinase C. The recent description and analysis of an agent (Bryostatin) which activates only protein kinase C did not produce the same spectrum of activity as previously documented with the phorbol esters and attributed to protein kinase C activation [28].

Investigations, therefore, have focused on the calcium-dependent antigenic signal in order to determine the point at which CsA exerts its effects. The evidence that CsA may act along this calcium-dependent pathway stems from a number of sources who have examined T-lymphocyte subsets in addition to the phorbol ester data. First, T-lymphocyte proliferation (CsA sensitive) requires a rise in intracellular calcium, whereas B-lymphocyte proliferation (CsA resistant) may progress in a calcium-independent fashion [29]. Second, it appears that lymphokine production by the T-helper cell is calcium dependent, whereas expression of interleukin-2 receptors may be calcium independent [30]. Third,

the inhibitory effect of CsA on T-lymphocyte proliferation appears to be potentiated by calcium channel blockers [31].

In initial studies, Palacios [32] hypothesized that CsA may compete for cell membrane receptors with mitogens (phytohemagglutinin, concanavalin A and the OKT3 monoclonal antibody) and alloantigen and, thus, block initial membrane events. In our laboratory, studies using a fluorescent derivative of CsA failed to demonstrate significant competition for binding sites between CsA and mitogens phytohemagglutinin, concanavalin A or the OKT3 monoclonal antibody, the anti-HLA-DR receptor antibody or anti-IL-2 receptor antibody. Fluorescent microscopy also demonstrated a rapid accumulation of fluorescent CsA in the cytoplasm of lymphocytes with little membrane or nuclear fluorescence [33]. Kay *et al.* [34] also concluded that CsA does not compete for membrane receptors, since A 23471, the calcium ionophore which is mitogenic by directly inducing a calcium flux, is much more sensitive than other mitogens to inhibition by CsA. They hypothesized that interference with membrane calcium fluxes could account for the CsA effect. Metcalfe [35], however, demonstrated subsequently that the secondary rise in intracellular calcium following mitogen stimulation (using concanavalin A) was not affected by CsA. We then began to investigate the probable effect of CsA on the next point in this activation sequence, i.e. at the level of calmodulin and other calcium-dependent proteins during cell activation. The activation of calmodulin, secondary to the rise in intracellular calcium, increases the activities of many enzymes within the cytoplasm of the cell, in particular calmodulin-dependent protein kinase, phosphorylases, phosphatases and enzymes involved in glucose metabolism and energy production. Calmodulin is also involved in cell cycle progression and gene expression. Additionally, calmodulin may play a regulatory role in cyclic nucleotide levels and prostaglandin synthesis and metabolism [36]. We found that CsA binds to calmodulin in a calcium-dependent manner, using fluorescent derivatives of both calmodulin and cyclosporine. Cyclosporine inhibits the ability of calmodulin to activate cyclic nucleotide phosphodiesterase, an enzyme critical in cyclic nucleotide turnover, in a dose-dependent fashion. At the cellular level, calmodulin inhibitors competitively inhibit a fluorescent cyclosporine from binding to intact lymphocytes [37]. In subsequent studies, we demonstrated the calcium dependence of a significant proportion of CsA binding to intact lymphocytes [38]. The addition of exogenous calmodulin completely overcomes the inhibitory effect of CsA on the mixed lymphocyte reaction [39]. LeGrue *et al.* [40] also demonstrated CsA binding to calmodulin using fluorometric techniques. They further demonstrated that CsA inhibits calmodulin-activated phosphodiesterase. In their assay, however, the IC_{50} for CsA was above 5 μM rather than the IC_{50} of 0.1 μM that we obtained. Fluorometric assays, however, fail to demonstrate significant differences in calmodulin binding by the derivatives of CsA that have different immunosuppressive capabilities [40]. This lack of specificity of the CsA derivatives toward calmodulin, using fluorometric techniques, may indicate a major drawback to this methodology. Conversely, a lack of stereospecificity may be characteristic with calmodulin inhibitors, as a class, and indicate that, for the CsA derivatives, immunosuppressive ability may be determined by other factors, including membrane partitioning or binding to other proteins in the cell.

In addition to this direct evidence for the specific interaction between CsA and calmodulin, a comparison between the effects of CsA and the effects of other calmodulin inhibitors reveals some similarities. Both CsA and calmodulin inhibitors interrupt the cell cycle at the G_1–S interface [22, 36]; create nuclear lobulation of lymphocytes, probably through an effect on the microtubular or microfilament cytoskeleton of the cells [41]; and cause renin release from the kidney and prolactin release from the posterior pituitary [42, 43]. CsA, the classic calmodulin inhibitors, and the calcium channel blockers, all will overcome the resistance of certain leukemia cell lines for vincristine, presumably by blocking calcium ATPase and drug efflux [44].

The classic calmodulin antagonists inhibit lymphocyte proliferation in the micromolar range, in a dose-dependent fashion, without direct cytotoxicity. In drug combination studies, these agents antagonize the immunosuppressive effect of CsA, indicating similar sites of action [45]. T-lymphocyte subset analysis has shown recently that the calmodulin inhibitor, W-7, much like CsA, inhibits interleukin-2 production and precursor cytotoxic T-lymphocyte generation, with little effect on suppressor cell generation [46].

Similarly, two peptide calmodulin inhibitors (mellitin and mastoparan) derived from insect venom inhibit lymphocyte proliferation in a dose-dependent fashion without cytotoxicity (unpublished data); there are also structural similarities between CsA and these peptide calmodulin inhibitors. These compounds are small cyclic oligopeptides with a sequence of hydrophobic amino acids in a beta-pleated sheet or alpha helix configuration. These peptides appear to bind to the calcium-dependent hydrophobic sites on the calmodulin molecule. By binding to and inhibiting calmodulin, CsA may also interrupt the normal propagation of the calcium–calmodulin-dependent first signals to the naive lymphocyte.

Peptide calmodulin inhibitors may also influence phospholipase A_2 and phospholipase C activity *in vitro*. These effects may be secondary to an indirect inhibition of calmodulin or other regulatory proteins or, more likely, may be a direct interaction with the phospholipases themselves. Our previous demonstration of cyclosporine's interaction with calmodulin and inhibition of calmodulin-dependent cell processes also implicates protein kinase C, phospholipase A_2 and/or phospholipase C as potential targets for inhibition or activation by cyclosporine. Using an affinity-labeled CsA derivative, we recently demonstrated that CsA binds to five cytoplasmic proteins of $<20{,}000\ M_r$ in lymphocytes, three of which cross-react with an anti-calmodulin antibody [47]. One of these proteins may be "cyclophilin", as CsA binding protein described by Handschumacher *et al.* [48]. These CsA binding proteins, including cyclophilin, may regulate other calcium-dependent

proteins within the cell including the phospholipases A_2 and C, which may be intimately involved in cell activation. Recently, it was demonstrated that CsA inhibits prostaglandin synthesis in rat peritoneal macrophages. This inhibition was abrogated by the addition of exogenous arachidonic acid. Using pancreatic phospholipase A_2 in an *in vitro* biochemical assay, it was shown that CsA directly inhibits this calcium-dependent protein [49].

A direct effect on macrophage phospholipase A_2 activity by CsA may indicate a point of functional overlap between CsA and steroids. Steroid hormones appear to act through regulation of lipomodulin synthesis. Synthesis of lipomodulin is increased following steroid administration. Lipomodulin binds and inhibits phospholipase A_2 [50]. The immunosuppressive action of the steroid hormones appears to potentiate the *in vivo* effect of CsA. *In vitro*, however, there is some overlap of the effects of CsA and the steroid hormones. It is conceivable that the combined activities of CsA and steroids toward macrophage phospholipase A_2 activity *in vitro* may account for the difficulty in separating their respective effects on T lymphocytes. It is also conceivable that CsA interacts with lipomodulin directly or with proteins that regulate lipomodulin.

Recent studies indicate that CsA may exert its *in vivo* effects on the pituitary axis to increase prolactin release and/or compete directly for prolactin receptors on T lymphocytes [51]. The theories are confusing in that this competition for receptors may activate or block a presumed prolactin effect on T lymphocytes that may be inhibitory or stimulatory depending on concentration and duration of prolactin administration. These data are also inconsistent with accumulating evidence of a cytoplasmic and/or nuclear site(s) of action for CsA at pharmacologic doses.

Our working hypothesis is that CsA is a peptide inhibitor of calmodulin. Many of its effects at the cell level can be directly or indirectly related to inhibition of calmodulin or other calcium-dependent proteins. Many of the dose-dependent toxic effects of CsA appear to be comparable to those seen with other peptide calmodulin inhibitors, variously described as "lysosomotropic drugs" or membrane active agents [52]. At toxic doses above 10 μg/ml, direct cytotoxicity is evident, possibly secondary to membrane damage. Between 1 and 10 μg/ml, general metabolic effects may be seen secondary to mitochondrial membrane damage and profound ATP depletion. Between 0.1 and 1 μg/ml, effects on macrophages and cytotoxic T-cell generation, including IL-2 receptor generation, may be prominent. At levels at, and below, 0.1 μg/ml, IL-2 production appears to be inhibited by CsA.

Future research directions should follow a number of avenues in attempts to outline completely the mechanisms of action and structure–activity relationships of CsA, as well as to utilize CsA to dissect T-lymphocyte activation events and subset generation. First, the specificity of the CsA derivatives for calmodulin should be addressed using binding techniques that are more sensitive than the previously used fluorometric techniques. Similarly, the relative hydrophobicity and membrane partitioning coefficient for the CsA derivatives should be determined. Second, knowledge of the structural relationships of CsA and its derivatives may allow for the design and development of other peptide inhibitors of calmodulin or derivatives of CsA with more specific immunosuppression and possibly less toxicity. Third, the interaction of CsA with the other calcium-dependent proteins should be investigated further using T lymphocytes, particularly their phospholipases A_2 and C. These interactions might give us a better understanding of the T-lymphocyte activation in the primary immune response. It may also reveal why some secondary immune responses are CsA resistant. Precise knowledge of these activation pathways will allow the clinician to select combinations of immunosuppressives to obtain the optimum effect. Fourth, the identification and cellular function of the potentially important, highly conserved protein, cyclophilin, and its role in T-lymphocyte activation, are crucial for our full understanding of the mechanism of action of CsA. Fifth, at the T-lymphocyte subset level, investigations will continue to identify the activation pathways for the maturation and expression of suppressor T cells that appear to be CsA resistant. CsA may thus be used as a tool to determine the relative importance of calmodulin, cyclophilin and other calcium-dependent proteins, such as protein kinase C and the phospholipases A_2 and C, in T-lymphocyte function.

CsA may also be used as a tool to examine the mechanism of chemotherapeutic drug resistance by tumor cells, a growing clinical problem in oncology; this mechanism appears to be linked to the calcium channels and calmodulin.

In summary, CsA is a novel immunosuppressive agent with a growing number of clinical uses and potential effects. At immunosuppressive non-cytotoxic doses, CsA may exert its effects on T lymphocytes and macrophages at the level of calmodulin and other calcium-dependent proteins. This action may explain many of the effects of CsA on tissues other than the cells of the immune system. CsA can be used also to examine other clinical problems such as drug resistance by tumor cells and as a tool to study T-lymphocyte activation events and subset generation.

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