Cyclosporine A, an In Vitro Calmodulin Antagonist, Induces Nuclear Lobulations in Human T Cell Lymphocytes and Monocytes

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Abstract. Cyclosporine A is a noncytotoxic, natural, 11 amino acid cyclic peptide used clinically as an immunosuppressant to prevent organ rejection after transplantation. Cyclosporine A is an in vitro calmodulin antagonist. At the low concentrations required to inhibit calmodulin-dependent phosphodiesterase in vitro, cyclosporine A causes a dramatic alteration in the nuclear morphology of 23% of human peripheral blood mononuclear leukocytes in vitro without loss of viability. The shape of the nucleus changes from ovoid to a distinctive, radially splayed lobulated structure. The changes occur in a dose-dependent manner in 60 min at 37°C. Specific monoclonal antibodies to human leukocytes identify the cells susceptible to nuclear lobulation by cyclosporine A as OKT4 antigen-positive T cell lymphocytes and monocytes. The lobulated nuclei are 2N as determined by flow cytometric measurement of ethidium bromide fluorescence of DNA. The cyclosporine A–induced lobulation of T cell nuclei requires both physiologic temperature and metabolic energy. Although structurally different than cyclosporine A, the calmodulin antagonists R24571 and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] also produce T cell nuclear lobulations that are indistinguishable from the nuclear lobulations caused by cyclosporine A. These data indicate that nonmitotic structural elements that govern normal nuclear morphology in a subset of mononuclear leukocytes appear to require a calmodulin-mediated process. Cyclosporine A may be a useful noncytotoxic inhibitor of calmodulin-dependent systems that influence nuclear structure and function.

CYCLOSPORINE A (CsA)[1] is a natural cyclic endecapeptide derived from the *Tolypocladium inflatum* fungus with profound clinical and experimental immunosuppressive properties. CsA is employed widely in the treatment of graft-versus host disease after bone marrow transplantation and for graft rejection in solid organ transplantation. This drug is now under investigation as a potential therapeutic agent in autoimmune disease, malaria, and schistosomiasis (2, 3). The mechanism by which CsA exerts its immunosuppressive action remains elusive. Recently CsA has been demonstrated to be a calmodulin-specific antagonist in the brain phosphodiesterase in vitro system (6). Furthermore, the calmodulin antagonists W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide] and R24571 prevent binding of CsA to T lymphocyte clones. CsA has a specific cytosolic 15K binding protein, cyclophilin; this highly basic receptor protein for CsA shows no NH_2 terminal amino acid sequence homology to any published protein sequence (10). Recently, cyclosporine A was found to selectively inhibit the normal accumulation of T cell growth factor or interleukin-2 mRNA in T lymphocyte cell lines stimulated by phorbol myristate acetate (9). These data suggest that the mechanism of action of CsA may be within the nucleus of the T cell, preventing transcription or causing posttranscriptional destabilization of message.

[1] *Abbreviations used in this paper:* CsA, cyclosporine A; dansCsA, a dansylated derivative of native CsA; NMI, nuclear morphology index; W-7 *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide.

CsA and a dansylated derivative of native CsA (dansCsA) with equivalent potency as an in vitro calmodulin antagonist are herein reported to alter the nuclear morphology of a subpopulation of human peripheral blood mononuclear leukocytes. The nuclear morphologic changes occurred at 37°C within 1 h of drug exposure but without apparent loss of cell viability as assessed by trypan blue dye exclusion.

The cells with nuclei susceptible to cyclosporine A–induced nuclear lobulation were identified as OKT4$^+$ (helper) T cells and monocytes. The following studies were undertaken to begin characterization of the effect of CsA on nuclear structure.

Materials and Methods

Human peripheral blood mononuclear leukocytes were obtained by Ficoll-Hypaque isolation and washed three times in Ca^{++}-free phosphate-buffered saline (PBS). CsA (OL-27-400) and dansCsA (205-797) were generous gifts of Sandoz Ltd. (Basel, Switzerland). Drug concentration was 1 μM in Ca^{++}-free PBS, pH 7.4, in all experiments. Stained with the fluorescent dansCsA, cells were sorted into dim and bright staining populations with the FACS-II analyzer (Becton Dickinson FACS, Oxnard, CA) using ultraviolet excitation (5, 11). Unfractionated peripheral blood mononuclear leukocytes were used in both the time-course and inhibitor experiments. To inhibit energy-dependent processes, cells were pretreated for 2 h at 37°C with dinitrophenol at 0.5 mg/ml of 2-deoxyglucose at 1 mg/ml. The calmodulin inhibitor R-24571 was obtained from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany), and W-7 was acquired from Sigma Chemical Co. (St. Louis, MO). Viability before and after CsA treatment was assessed by trypan blue dye exclusion in early experiments; this consistently yielded >95% dye impermeable cells. Cells were prepared for nuclear morphometrics by cytocentrifugation. Wright-Giemsa staining immediately followed centrifugation at the conclusion of each incubation. To determine whether CsA-induced nuclear lobulation was influenced by DNA replication, leukocyte nuclear DNA content was assessed by ethidium bromide fluorescence using a FACS-II cytofluorimeter (17).

To quantitate nuclear shape change, morphometrics were performed with computer-assisted image analysis, extensively described elsewhere (8). Nuclear image analysis was performed under oil immersion using a Zeiss planapochromat 100× objective and a 12.5× ocular. With an affixed Zeiss camera lucida (model 47462) and a Hewlett-Packard 9874A digitizer pad (Hewlett-Packard Co.), nuclear perimeter was traced using a small green light-emitting diode (model 276-037, Radio Shack Div., Tandy Corp., Fort Worth, TX) attached to the digitizer puck. A Hewlett-Packard 9825 digital computer was calibrated using a Zeiss stage micrometer graduated in 0.1-mm units. From points generated by nuclear tracing, the computer calculated nuclear perimeter, nuclear cross-sectional area, and a nuclear morphology index (NMI) for each nucleus. The NMI is calculated from two computed radii: Rc, the radius computed for a circle with a circumference (C) equal to the traced nuclear perimeter; and rA, the radius computed for a circle with an area (A) equal to the traced nuclear cross-sectional area. Thus, where C is the circumference and A is area. $(Rc/rA) = C/2\pi/(A/\pi)^{1/2}$ of the digitizer traced nuclei. A perfectly circular nucleus would have a Rc/rA of 1.000.

In this system with 10 successive tracings, a geometrically accurate circle of known area of 30 μm^2 and an Rc/rA of 1.000 can be computed to have an area of 30.5 $\mu m^2 \pm 0.1$ SD and an Rc/rA of 1.006 ± 0.001 SD (13). To express deviation from nuclear circularity, NMI units are expressed as $(Rc/rA - 1.000) \times 10^3$. A perfectly circular nucleus would have an NMI value of zero. As the NMI value increased it denoted increasing deviation from circularity, i.e., lobulation. 100 nuclei were digitized from each cytocentrifuged slide of peripheral blood leukocytes, and the mean and SEM of NMI were calculated.

The immunocytochemistry studies used methods similar to those described by Martin et al. (13). Cell suspensions were counted and checked for viability. The cell count was adjusted to ~5×10^4/ml and polyethylene glycol was added to a final concentration of 10%. The cells were attached to glass slides using the Shandon cytospin (Shandon Southern Instruments Inc., Sewickley, PA) at 700 rpm for 5 min. The slides were air dried and fixed with acetone. The monoclonal antibodies used included HLe1, Leu1, and Leu12 (Becton-Dickinson & Co., Sunnyvale, CA), OKT11, OKT4, OKT8 and OKM1 (Ortho Diagnostics, Raritan, NJ), B1 (Coulter Electronics Inc., Hiahleah, FL), and anti-Tac (kindly provided by Dr. T. Waldmann, National Institutes of Health). Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) was used as the second antibody. In addition, biotin-conjugated goat anti-mu heavy chain (Tago Inc., Burlingame, CA) was used for surface IgM. The dilutions of the primary antibodies varied from 1:20 to 1:100, depending on the antibody lot.

The avidin-biotin complex staining methods have been described previously (1, 12). The slides were again fixed with acetone and washed with PBS containing 0.2% bovine serum albumin. After the slides were washed they were incubated for 30 min with the monoclonal antibody and washed again. Negative controls were incubated with unrelated antibody. Endogenous peroxidase was inactivated with methanol and hydrogen peroxide. The section antibody, biotinylated horse anti-mouse IgG (1:50), was layered for 30 min and the slides were washed. The sections were exposed to the avidin-biotin complex (30 min), developed with diaminobenzidine (25 min or as indicated by HLe1) and copper sulfate (5 min), and counterstained with a fast Giemsa stain.

Results

CsA and dansCsA caused profound changes in mononuclear leukocyte nuclear morphology without loss of viability as assessed by trypan blue dye exclusion (Fig. 1). The ovoid nuclei became distinctively lobulated by CsA. The affected nuclei appeared by light microscopy as splayed lobules attached to a 1–2-μm axle of central chromatin. As many as 20 separate lobes could be observed in one nucleus in some of the drug-treated cells.

The dramatic lobulation of nuclear morphology by dansCsA appeared in a subpopulation of mononuclear leukocytes in peripheral blood. Nuclear lobulation provoked by dansCsA was evident in 21 ± 9% (mean ± SEM) of cells as opposed to the untreated control group, which had 3 ± 1% (mean ± SEM) of cells meeting the criterion of three or more nuclear lobulations.

The striking nuclear lobulation of mononuclear leukocytes was induced by dansCsA in a dose-dependent manner (Fig. 2). Structurally dissimilar calmodulin inhibitors R24571 and W-7 also caused human leukocyte nuclear lobulations in identical incubation conditions. The concentrations of drug required for half-maximal induction of nuclear lobulation for dansCsA, R24571, and W-7 all fall in the $1–2 \times 10^{-7}$ M range, although dansCsA induced twice as many cells to undergo lobulation. This drug concentration range for W-7 and dansCsA has been shown to be sufficient for competitive inhibition of calmodulin-dependent phosphodiesterase (6). The concentration of dansCsA to give a half-maximal number of cells with lobulated nuclei is remarkably comparable to the K_D for dansCsA binding to calmodulin in vitro (2×10^{-7} M) and the K_D of CsA binding T lymphocytes ($2–3 \times 10^{-7}$ M) (4, 6, 15).

A twofold enrichment of mononuclear leukocytes that undergo nuclear lobulation was achieved by dansCsA staining and sorting through a FACS-II system. The use of the fluorescent CsA probe allows for the isolation of T cells that have a differential binding capacity for CsA; of these, cells are either intensely or weakly fluorescent (5). Although comprising only 18% of the human peripheral blood mononuclear leukocytes, the bright-staining cells contained 95% of the lobulated nuclei observed in both bright and dim populations. The bright population has been shown to contain B lymphocytes, OKT4+ and OKT8+ T lymphocytes, and monocytes (5). Drug uptake assessed by high dansCsA fluorescence, however, is not sufficient alone to induce lobulation, as 43% of the bright population nuclei were not lobulated.

The kinetics of CsA-induced lobulation were characterized by time-course experiments using computer-assisted morphometrics (see Materials and Methods) (Fig. 3). At 1.0 μM concentration, dansCsA caused a 15-fold increase in mononuclear leukocyte mean NMI. The maximum effect of lobu-

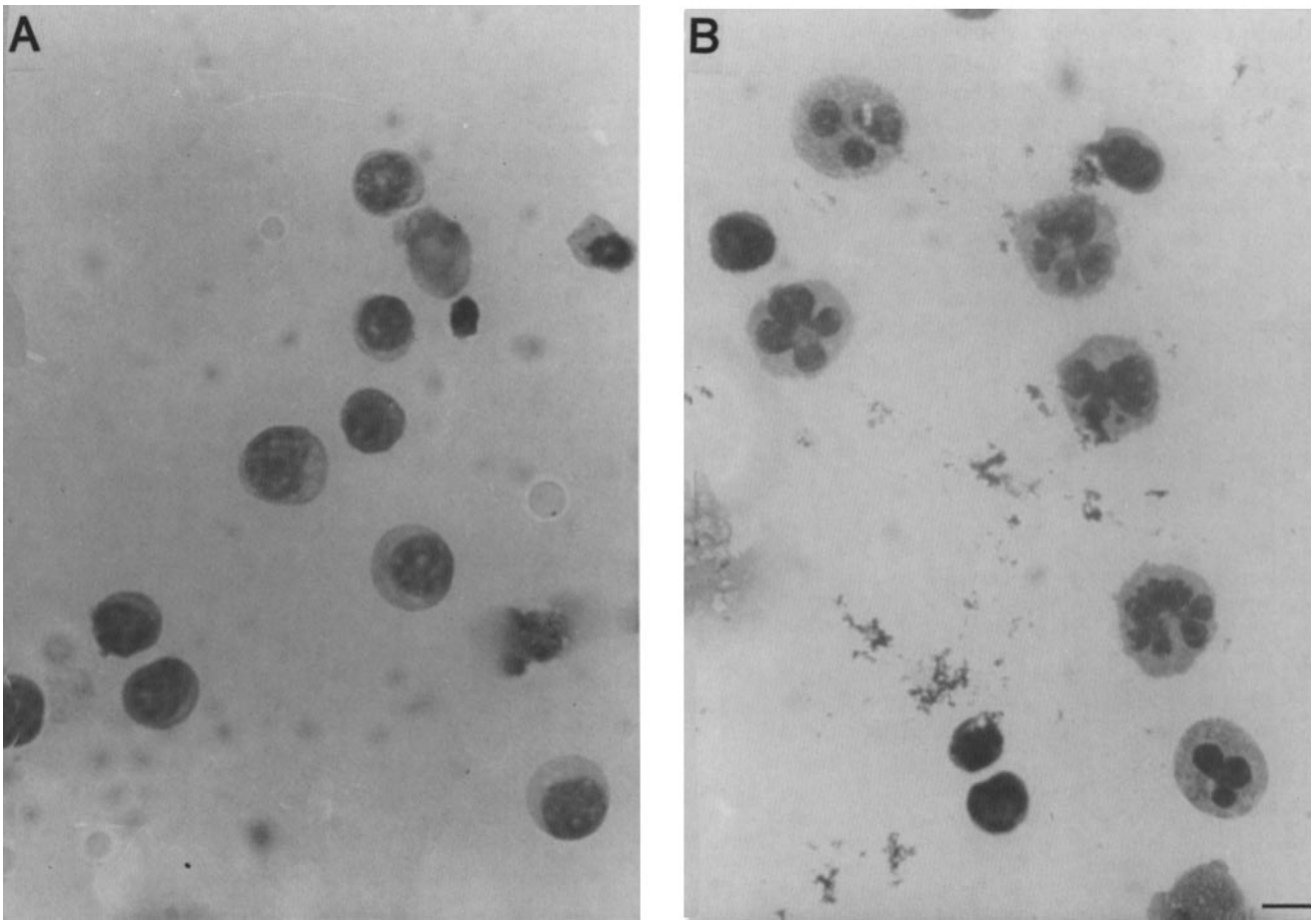

Figure 1. Photomicrographs comparing untreated and treated human mononuclear leukocyte nuclei. Human peripheral blood mononuclear leukocytes were incubated in PBS at 37° for 1 h in the absence (*A*) and presence (*B*) of 1 μM CsA. Cells were >95% viable by trypan blue dye exclusion at the conclusion of the experiment. Leukocytes were stained by Wright Giemsa after cytocentrifugation. Bar, 8 μm.

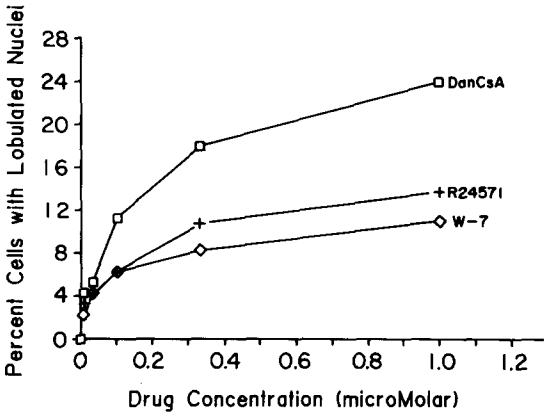

Figure 2. Dose response of dansCsA, R24571, and W-7 on the induction of nuclear lobulation in peripheral blood mononuclear cells. Cells were incubated for 1 h at 37°C in various concentrations of drugs before cytocentrifugation and staining. The concentrations of each drug yielding half-maximal percent of cells with nuclear lobulations were calculated by regression analysis; the values for dansCsA, R24571, and W-7 were 0.12, 0.15, and 0.18 μM, respectively.

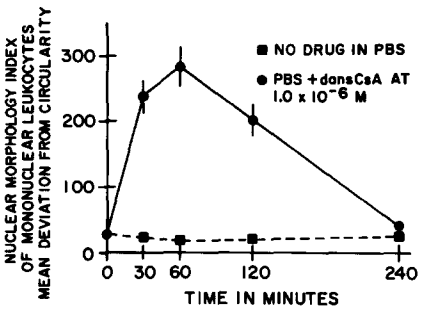

Figure 3. Time course of nuclear lobulation caused by CsA. Unfractionated human mononuclear leukocytes were incubated with 1 μM dansCsA and assessed for changes in nuclear morphology at various time points. NMI is expressed in units of $(Rc/rA - 1.000) \times 1{,}000$ (see Materials and Methods). The perimeters of 100 nuclei were digitized through computer-assisted morphometrics, and mean NMI and SEM were computed at each time point. Viability by trypan blue dye exclusion was >95%.

lation was observed at 60 min. Over 80% of the alteration in NMI index of mononuclear leukocytes treated with dansCsA occurred within the first 30 min of treatment. Despite the prominent changes in nuclear morphology, cell viability as assessed by trypan blue exclusion was greater than 95%. CsA-provoked nuclear lobulation appeared to be transient, as NMI returned to the untreated value by 4 h. Incubation in PBS alone, however, did not cause any significant changes.

To determine whether or not susceptibility to nuclear lob-

ulation by CsA might be influenced by chromosomal replication, ethidium bromide fluorescence of nuclei in the brightly and dimly dansCsA-staining leukocytes was compared (Fig. 4). Whereas 45% of the brightly dansCsA-stained cells had lobulated nuclei, the FACS-II histographic distribution of nuclear DNA content was 2N. A 2N distribution of nuclear ethidium bromide fluorescence was found in the unlobulated nuclei of the dimly stained cells as well. Thus nuclear lobulation by CsA did not appear to reflect a capability special to nuclei entering mitosis.

The effects of temperature, metabolic energy inhibitors, and calmodulin inhibitor R24571 on the extent of alteration of nuclear morphology by dansCsA were evaluated at 60 min of treatment, when the nuclear change was maximal (Fig. 5). The extent of lobulation, induced by CsA as determined by NMI, was almost identical to that induced by dansCsA at 37°C. Microscopically, the lobulated mononuclear leukocyte nuclei induced by CsA and dansCsA were indistinguishable from each other. The kinetics of nuclear morphologic index changes in the presence of CsA appear identical at 0, 30, and 60 min to those of dansCsA at 37°C in Fig. 3 (data not presented), indicating that the dansyl groups on dansCsA do not influence the nuclear lobulation in themselves.

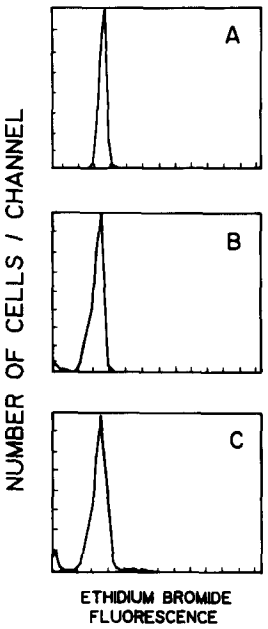

Figure 4. FACS II histogram plots of 2N ethidium bromide DNA fluorescence distribution in dansCsA-treated human mononuclear leukocytes. (*A*) Human peripheral blood leukocytes were stained with 1.0 μM dansCsA and assessed directly for ethidium bromide DNA fluorescence (see Materials and Methods). Leukocytes were FACS-II sorted into (*B*) a dim dansCsA fluorescence population and (*C*) a bright dansCsA fluorescence population. The dim and bright dansCsA-stained leukocytes were then assayed for ethidium bromide DNA fluorescence. The total numbers of cells counted for *A–C* were 14,871, 14,901, and 14,895, respectively.

Nuclear lobulation by CsA showed temperature dependence. Incubation of mononuclear leukocytes at 4°C in 1 μM CsA prevented any change in nuclear morphology. In addition to being temperature dependent, CsA-induced nuclear lobulation apparently also required metabolic energy. Pretreatment with the mitochondrial uncoupler dinitrophenol and 2-deoxyglucose for 2 h in PBS caused a 60% inhibition in nuclear lobulation after a 1-h incubation in CsA at 37°C. Complete abolition of CsA-induced nuclear lobulation in the sensitive leukocyte subpopulation occurred in the presence of the metabolic inhibitor sodium azide at 0.1% (data not shown).

The calmodulin inhibitor R24571 at 1 μM caused an increase in mean NMI slightly greater than that caused by CsA or dansCsA in 1 h at 37°C. By light microscopy, the nuclei lobulated by R24571 mononuclear leukocytes were similar to those treated with CsA. Viability of leukocytes at 1 h at 37°C was 82% as assessed by trypan blue dye exclusion in the presence of R24571 as opposed to >95% viable in CsA at the same concentration.

To assess the phenotypes of cells demonstrating lobulated nuclei, peripheral blood lymphocytes incubated with CsA (60 min, 37°C) were stained (immunoperoxidase) with a panel of monoclonal antibodies. The results shown in Table I demonstrate that most (70%) cells demonstrating lobulated nuclei were T lymphocytes that expressed the OKT4+ antigen. The remaining cells that showed CsA-induced lobulated nuclei

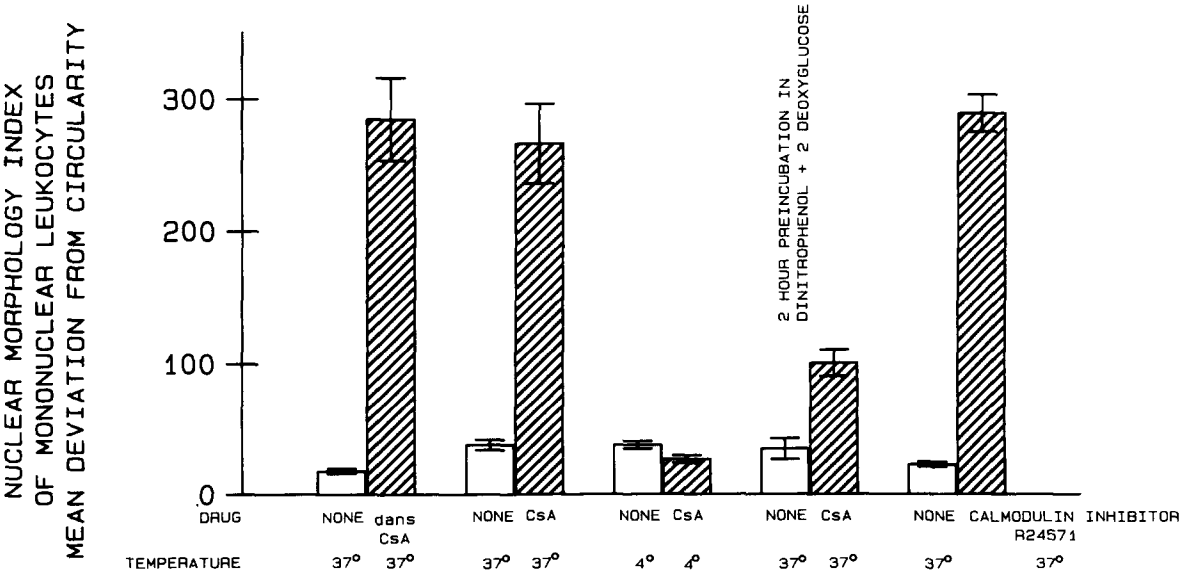

Figure 5. Nuclear lobulation at 60 min: comparison of temperature and drug effects. Mean NMI standard errors for 100 nuclei in each condition were computed as described. The following concentrations were used: dansCsA and CsA at 1 μM in PBS; 1 h of pretreatment with dinitrophenol and 2-deoxyglucose at 0.5 mg/ml and 1 mg/ml, respectively; and calmodulin inhibitor R24571 at 1 μM.

*Table I. Immunologic Phenotypes of Arborized Cells**

Antibody	Cell type	Subject 1	Subject 2	Subject 3
HLe1	Leukocyte	100	100	100
Leu1	Pan-T	70	70	85
OKT11	Pan-T, SRBC	70	75	80
OKT4	T helper cell	70	70	75
OKT8	Cytotoxic/supp.	<5	15	<5
B1	Pan B	<5	<5	<5
Leu12	Pan B	<5	<5	<5
IgM	SIg, B cell	<5	<5	<5
OKM1	Monocyte/gran.	30	40	30
Tac	I12 receptor	ND	<5	<5

SRBC, sheep erythrocyte; supp., suppressor; gran., granulocyte; SIg, surface immunoglobulin.

* Peripheral blood lymphocytes were incubated with 1 μM CsA for 1 h at 37°C before immunoperoxidase staining.

were monocytes, as demonstrated by morphologic examination and by staining with the OKM1 monoclonal antibody.

Discussion

CsA and dansCsA rapidly induced lobulation of the nuclei of a subpopulation of human peripheral blood mononuclear leukocytes in vitro in a dose-dependent manner. This striking alteration in nuclear morphology induced by CsA did not affect cell viability, as assessed by trypan blue dye exclusion. The lobulation of the nuclei required both physiologic temperature and metabolic energy in Ca^{++}-free PBS. Leukocyte viability in the presence of 1.0 μM CsA is consistent with the known noncytotoxic immunosuppressive properties of CsA. The subpopulation of leukocytes susceptible to nuclear lobulation by CsA was identified by specific monoclonal antibody analyses as $OKT4^+$ T cell lymphocytes, the T helper subset responsible for interleukin-2 production. However, not all $OKT4^+$ lymphocytes demonstrated lobulated nuclei since the dim staining population also contained this T cell subset, which did not show lobulated nuclei. Cellular drug uptake alone was not sufficient to induce nuclear lobulation, as 43% of the brightly fluorescent leukocytes treated with dansCsA maintained an ovoid nuclear morphology.

CsA induces nuclear lobulation at a concentration sufficient to inhibit calmodulin-specific phosphodiesterase activity in vitro (6), implicating a calmodulin-dependent process in nuclear lobulation. Furthermore, the calmodulin antagonists R24571 and W-7, while structurally different, have similar effects on the degree of nuclear lobulation, as assessed by computer-assisted image analysis. Although the calmodulin inhibitors W-7, R24571, and CsA all have nearly identical dissociation constants, CsA is twice as efficient as W-7 and R24571 in inducing nuclear lobulation after treatment. The efficiency of CsA in inducing nuclear lobulation, putatively through inhibition of calmodulin-dependent processes, may indicate that CsA is a more efficient antagonist or that its properties are unique relative to those of other calmodulin antagonists. It will be important to study the relationship of CsA binding to calmodulin and identify what calmodulin-dependent functions are inhibited by this agent as compared with other calmodulin inhibitors.

Calmodulin antagonists have been shown previously to induce structural problems within the nucleus. Treatment with the potent calmodulin antagonist trifluoroperazine can induce intranuclear paracystalline bundles of actin to form in HeLa cells (14). Trifluoroperazine also causes dramatic crenelations in HeLa cell surface morphology, loss of cell spreading, and cell locomotion, all of which are consistent with derangement of calmodulin-dependent microfilament organization. In addition, calmodulin has been reported to be associated with the cytoplasmic microtubular complex and the centrosomes (7). Recent studies have indicated that the interactions of calmodulin with these cytoskeletal elements regulate the Ca^{++}-dependent depolymerization of these elements (7). Lobulation of nuclei due to inhibition of calmodulin-dependent cytoskeletal elements has not been reported previously. The lobulated nuclei induced by CsA or by the calmodulin antagonists appear to splay chromatin radially from a central hub which may reflect novel changes in the mechanical integrity of nuclear structural elements. The chemomechanical mechanism of viable nuclear lobulation through calmodulin inhibition will require further experimental dissection. However, the nuclear lobulations in T cells and monocytes may reflect mechanically interlocked cytoskeletal and nuclear skeletal elements that are calmodulin regulated. Myosin light chain kinase, Ca^{++}-dependent calmodulin binding protein, and calmodulin itself are mandatory cytoplasmic components for contractility in nonsmooth muscle and are identifiable components of the nuclear matrix (16).

The effect of CsA on nuclear morphology in relation to its immunosuppressive activity remains unclear. However, this action of CsA may cause changes in the cytoplasmic-to-nuclear signals necessary for T cell activation. CsA may prove to be a useful new noncytotoxic pharmacologic probe against calmodulin-dependent chemomechanical processes that influence nuclear structure.

We acknowledge the technical assistance of Pamela Engel, Cathy Mercaldi, Sofia Taylor, Karen L. Dattilio, and Donna L. Suresch. J. W. Simons is a Henry Strong Denison Fund Scholar of The Johns Hopkins University School of Medicine; A. D. Hess is a scholar of the Leukemia Society of America, Inc.

This work was supported by grants CA 15396, AI 20990, and AI20173 from the National Institutes of Health, ACS Im 398 from the American Cancer Society, and by a gift from the Harley W. Howell Foundation.

Received for publication 27 March 1985, and in revised form 27 September 1985.

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