Physical characterization of cyclosporine binding sites in lymphocytes

Charles D. Niebylski and Howard R. Petty
Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202 USA

ABSTRACT The binding of cyclosporine to human peripheral blood lymphocytes (PBLs) was studied by measuring the fluorescence emission spectrum and lifetime of the fluorescent and immunosuppressive cyclosporine derivative dansyl-cyclosporine (DCs). The emission maximum and fluorescence lifetime of DCs were characterized in several solvents. The fluorescence emission maximum and lifetime of DCs increased at a high dielectric constant. The fluorescence lifetime decay curve of DCs was a monoexponential function in all solvents tested. Fluorescence micrographs of lipid vesicles and erythrocytes labeled with DCs exhibit uniform staining patterns, whereas PBLs show heterogeneous DCs labeling. DCs exhibits a relatively low emission maximum (490 nm) in erythrocyte membranes. Such an emission maximum is characteristic of a hydrophobic environment. DCs in PBLs also has a low emission maximum (484 nm). The lifetime of DCs in PBLs required two exponential terms to properly fit the lifetime decay curve and could not be attributed to light scattering. One short component (4.7 ± 1.0 ns) and a second long component (18.5 ± 1.0 ns) were resolved from the DCs fluorescence decay curves. Time-resolved anisotropy of DCs in PBLs revealed that the labeled drug was present in an anisotropic environment, consistent with at least some DCs being bound to a membrane. These fluorescence studies suggest that DCs interacts with multiple and/or heterogeneous sites in peripheral blood lymphocytes.

INTRODUCTION

Cyclosporine A (CsA) is an immunosuppressive agent (1) widely prescribed to prevent graft rejection (2, 3). CsA interferes with an early event in T cell activation (4–6). Unfortunately, CsA's mechanism of action remains unknown (7). Since CsA is hydrophobic, it would be expected to partition into membranes. Evidence suggests that the plasma membrane is the first site of CsA action. Changes in phospholipid metabolism of lymphocyte membranes have been shown to follow CsA exposure (6, 8). Other investigators have detected lymphocyte membrane depolarization after addition of CsA (9, 10). Raman spectroscopic and calorimetric studies have shown that CsA lowers the partition coefficient and the heat of activation of artificial membranes (11, 12). CsA has been shown to bind to a cytosolic lymphocyte protein, termed cyclophilin, that is closely associated with calmodulin (13, 14). Both tritiated CsA and the fluorescent CsA derivative dansyl-cyclosporine (DCs) retain immunosuppressive activity similar to that of native CsA, thus making them valuable tools in the study of CsA activity (14, 15). Fluorescence microscopy of DCs has shown that this drug is localized at the plasma membrane as well as in intracellular pools (16).

Fluorescent molecules are very sensitive to their environment. The fluorescence emission maximum and fluorescence lifetime can be used to measure physical differences in a probe's environment, such as hydrophobicity (17). Careful study of changes in the fluorescence DCs should provide valuable information concerning cyclosporine binding sites. In this study the fluorescence properties of DCs in several known solvent systems of differing hydrophobicity are compared with those of DCs in various membrane/cell systems, including peripheral blood lymphocytes. From these data, it is possible to characterize in biophysical terms the local environment of DCs in cells. Additional evidence concerning the binding and rotational motion of DCs is provided by time-resolved fluorescence anisotropy studies.

Address correspondence to H. R. Petty.

MATERIALS AND METHODS

Cells

Human erythrocytes and peripheral blood lymphocytes (PBLs) were obtained from normal subjects. PBLs were isolated by step-density gradient centrifugation using Ficoll-Hypaque solutions (Sigma Chemical Co., St. Louis, MO). Lymphocytes were enriched by allowing monocytes to adhere to plastic dishes for 30 min at 37°C. The size, refractivity, and Geimsa staining indicated that the nonadherent population was >90% lymphocytes. Lymphocytes were >96% viable as determined by Trypan Blue exclusion. Cells were studied at a concentration of 2×10^5 cells/ml.

Liposomes

Artificial membranes were prepared from dimyristoylphospatidylcholine (DMPC). Anhydrous phospholipids (>95% pure) were obtained from Sigma Chemical Co. and then dissolved in chloroform at millimolar concentrations. The lipid solutions were dried in 5-ml round-bottom flasks using a Buchi rotavapor. One milliliter of phosphate-buffered saline (PBS) or Hank's buffered saline solution (HBSS) (pH 7.4) was added to the flask. The flask was agitated vigorously after hydration above the phase transition temperature. These large multilamellar liposomes were prepared weekly and stored under argon gas at 4°C.

Solvents and fluorophores

Technical grade solvents (chloroform, acetone, ethanol, and methanol) were obtained from Fisher Scientific (Pittsburgh, PA). DCs ((n-dansyl)-o-lysyl-8-cyclosporine) was the generous gift of Drs. Roland Wenger, D. Römer, and E. Rissi (Sandoz, Ltd., Basel, Switzerland). Dansylamide (5-[dimethylamino]naphthalene-1-sulfonamide) was obtained from Sigma Chemical Co. DCs was prepared at 0.01 M in absolute ethanol and diluted in HBSS at pH 7.4 to a final working

concentration of 1–5 × 10^{-6} M (0.05% ethanol). For solvent experiments, DCs (0.01 M in EtOH) was diluted in the appropriate solvent to a final concentration of 5 × 10^{-6} M. In tests of DCs in HBSS, 0.1% Triton-X100 detergent (Sigma Chemical Co.) was added to minimize the formation of DCs aggregates.

Fluorescence microscopy

Cells were examined in a Zeiss Axiovert 35 inverted fluorescence microscope (Carl Zeiss, New York, NY) interfaced with a Perceptics Biovision system (Knoxville, TN). Differential interference contract images were collected with a Dage-MTI charged-coupled device camera (Michigan City, IN). A Zeiss 100X (N.A. = 1.3) objective and a condenser of N.A. = 0.55 were used. DCs was excited using a mercury vapor lamp and emission detected with a 419 nm emission filter. A Dage-MTI model 66 SIT camera received the fluorescence image. Video signals were recorded on hard disk, streaming tape, or a Sony 5850 video recorder. Images were photographed with a Polaroid (Boston, MA) freeze-frame video recorder.

Fluorescence emission spectroscopy

Measurements of fluorescence emission spectra were performed with an MPF-66 spectrofluorometer (Perkin-Elmer, Norwalk, CT) equipped with a water bath. DCs was excited with 360 nm light (slit width = 7.5 nm), and the resulting emission spectra (slit width = 10 nm) were recorded. All emission spectra (n = 5 for all solvents) were reproducible with less than a 1.0% standard error in the emission maxima. 5 × 10^{-6} M DCs in the appropriate solvent was measured after a 45-min equilibration period. Erythrocytes and lymphocytes were incubated with 1–5 × 10^{-6} M DCs for 1 h at 37°C and washed twice with PBS at 37°C before analysis.

Fluorescence lifetime analysis

Fluorescence lifetimes of DCs were measured using a fluorescence lifetime instrument (Photochemical Research Associates, Inc., London, Ontario, Canada) using the single photon counting method for fluorescence lifetime determination (17). Excitation was provided by a thyratron-gated arc lamp containing 75–85 kPa of N_2 operating at 30 kHz with a 3.5–4.0 kU potential. The 337-nm N_2 emission line was selected by a grated monochromator and used to excite DCs. The appropriate fluorescence emission for each sample (as determined from the emission spectra) was selected by a second grated monochromator. Pulse width (full width at half maximum) was 2.0–2.5 ns for all samples. Scattering was <3% for all samples. Data were acquired at ≤2% of the lamp repetition rate. Data containing 4–9 × 10^3 peak channel counts and a 10^3-fold decay were collected in 512 channels using a multichannel analyzer. Samples were maintained at a constant temperature using a circulating water bath. For each decay curve, a corresponding lamp profile using a scattering solution (latex beads, milk, and/or unlabeled cells) was collected. Computer analysis deconvoluted the lamp curve from the sample curve, and the lifetime was then calculated from the resulting exponential decay curve. The cell concentration was 200,000 cells/ml for all experiments.

Time-resolved fluorescence anisotropy

Time-resolved fluorescence anisotropy (TRFA) measurements were made using the fluorescence lifetime instrument described above. TRFA studies used a Glan-Thompson cell for selecting polarization of excitation light and a Polaroid sheet for selecting the emission polarization (18). Briefly, two sets of data and lamp curves were collected for the vertical (v) and horizontal (h) polarizer positions specified in Eq. 1. The time dependence of the fluorescence anisotropy, $r(t)$, is given by

$$r(t) = \frac{I_{vv}(t) - I_{hv}(t)Q}{I_{vv}(t) + 2I_{hv}(t)Q} = \frac{d(t)}{s(t)}, \quad (1)$$

where $d(t)$ is the difference curve and $s(t)$ is the sum curve. Q is a total scaling factor that corrects for the intensity of excited light transmitted at different polarizer positions and variations in the number of photons per pulse emanating from the lamp during the experiment. This factor is

$$Q = GKI_{cr}, \quad (2)$$

where $G = I_{vh}/I_{hh}$ and $K = I_{hv}/I_{vv}$. The factor I_{cr} is the integrated count ratio; the total number of counts for each curve $[I_{vv}(t)/I_{hv}(t)]$ are obtained.

The angle (Θ) between the emission dipole and polar axis of DCs was calculated using the following equation (17):

$$r_\infty = \frac{3\cos^2\Theta - 1}{2}, \quad (3)$$

where r_∞ is the limiting anisotropy measured from the TRFA data. The derivation of Eq. 3 assumes that the absorption and emission dipoles are colinear. This angle, Θ, corresponds to the amount of wobble of a membrane-bound fluorophore (19).

RESULTS

Photomicroscopy

Photomicroscopy of DMPC vesicles stained with 3 × 10^{-6} M DCs for 1 h at 37°C shows that the fluorophore is uniformly distributed in the lipid bilayer (Fig. 1, *A* and *B*). Cyclosporine concentrations of <10^{-5} M were used in this study due to the formation of micelles at the critical micelle concentration of 3 × 10^{-5} M (8). A similar distribution pattern is found for human erythrocytes (Fig. 2, *A* and *B*). These results suggest an equivalent partitioning of DCs among these lipid membranes. Peripheral blood lymphocytes exhibit a brighter and more heterogeneous staining of cells (Fig. 2, *C–F*). Some cells show a uniform membrane-associated DCs staining, whereas others exhibit clustering and punctate fluorescence of the probe. These DCs-labeling patterns may be associated with plasma membrane binding and an intracellular compartment(s) or binding site(s) (10).

Fluorescence emission spectroscopy

Fluorescence emission spectra of DCs in various solvents show a marked difference in emission maxima (Table 1). The hydrophobicity of a solvent is related to its dielectric constant. Dielectric constants of the solvents used (20) are as follows: acetone (20.7), methanol (32.6), ethanol (24.3), chloroform (4.8), and water (80.4) (these values are the low frequency static values at 20–25°C). As Table 1 shows, the emission maxima of DCs is affected by the dielectric constant of the solvent. This physical property of DCs can be used as an indicator of the hydrophobicity of its environment.

The emission spectra of DCs is similar in erythrocytes

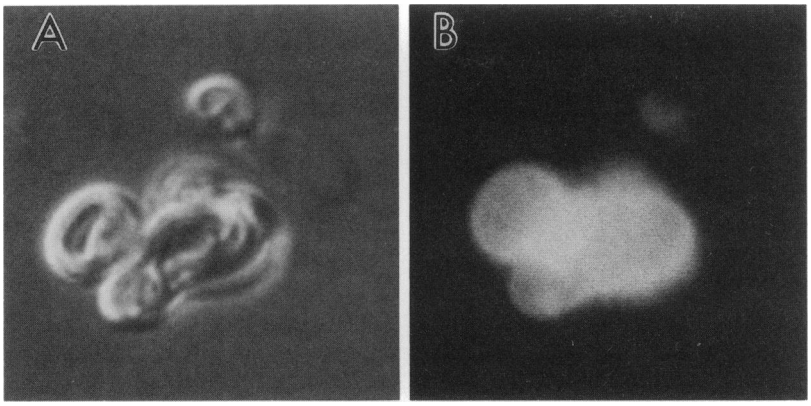

FIGURE 1 Photomicrographs of DMPC multilamellar liposomes labeled with DCs. DMPC liposomes were incubated with 3×10^{-6} M DCs at 37°C for 2 h and analyzed. (*A*) Differential interference contrast image of the liposomes. (*B*) Fluorescence image of the DCs in the artificial membranes. DCs stain DMPC liposomes in a bright and uniform manner. (×1,000)

and PBLs. A single broad peak with a maxima at 490 nm was found for DCs in red blood cells. A similar fluorescence emission spectrum was measured for DCs in PBLs (Fig. 3). This blue shift of the DCs emission is similar to the blue shifts observed for other membrane-bound fluorophores (19). Similar results (λ_{em} = 279 nm) were obtained using DMPC vesicles. The low wavelength emission maxima correspond to an environment that has a low dielectric constant (i.e., chloroform: λ_{em} = 490 nm, ϵ = 4.8), which suggests that its environment is largely hydrophobic.

Fluorescence lifetime measurements

A probe's fluorescence lifetime is also dependent on the polarity of its environment. As seen in Table 1, the fluorescence lifetimes of DCs in nonpolar solvents are not readily distinguished from one another as a function of dielectric constant (13.0–14.5 ns; n = 5). Distilled water and HBSS increased the fluorescence lifetime of DCs (17.6 ± 2.1 ns; n = 8), which is characteristic of an aqueous environment.

Computer analyses of fluorescence decay curves use the least-squares method of interpreting the data (21). Several statistical tests were used routinely in the analysis of each exponential fit. In determining whether a single- or double-exponential fit is required, the Durbin-Watson (DW) test parameter was evaluated in each case. The DW test parameter is sensitive to small nonrandom patterns in the residuals of an exponential fit. Such a parameter can determine whether a single or double exponential is required in fitting a fluorescence decay curve. This statistical test is more sensitive than the χ^2 to nonrandom residual patterns. Acceptable DW parameters for single- and double-exponential curve fits are >1.65 and >1.75, respectively. Statistical analysis of the single exponentials determined for DCs in all solvent and aqueous buffer runs gave acceptable DW and χ^2 values (ranges were DW = 1.82–2.03, χ^2 = 1.05–1.14). The fluorescence decay of DCs in erythrocytes was analyzed using both single- and double-exponential curve fitting. The single-exponential lifetime was 13.2 ± 2.3 ns (n = 3, DW = 1.35, χ^2 = 1.70), whereas the double-exponential fit gave τ_1 = 1.3 ± 0.5 ns and τ_2 = 13.9 ± 4.1 ns, with amplitudes of 0.09 and 0.05, respectively (DW = 2.11, χ^2 = 0.88). The fluorescence lifetime of DCs in lymphocytes was different from that of the solvent systems and erythrocytes (Fig. 4). Two lifetime components were required to fit the fluorescence decay curve of PBLs because single-exponential curve fitting of DCs lifetime decays resulted in poor statistical correlation (DW = 1.02 and χ^2 = 2.09). The double-exponential curve fit resulted in a fast component (τ_1 = 4.7 ± 1.0 ns) and slow component (τ_2 = 18.5 ± 1.0 ns) with amplitudes of 0.016 and 0.036, respectively (n = 9, DW = 2.01, χ^2 = 1.01). Similar double-exponential decays were obtained using a lymphocyte-like cell line. The fast component (τ < 8 ns) could not be observed in DMPC vesicles using single- or double-exponential curve fitting, thus suggesting that it is specific for cells.

Since the double-exponential curve fitting was only required in the presence of cells, it seemed possible that light scattering from the cell samples might have affected the lifetime measurements. To control for this possibility, the reagent dansylamide (τ = 3.31 ± 0.013 ns in 100% ethanol; DW = 1.8, χ^2 = 1.17), which would not be expected to specifically interact with cells, was used. Sample turbidity was raised using both intact cells and 0.6-μm latex beads (Polysciences Inc., Warrington, PA). Both of these types of control experiments yielded single-exponential decays with DW parameters of 2.0 or greater. Therefore, the required two-component fit was not due to the label per se or light scattering artifacts introduced by the cells. Furthermore, the lifetime heterogeneity likely was not due to DCs self-quenching since our experiments were conducted at low, physiologically

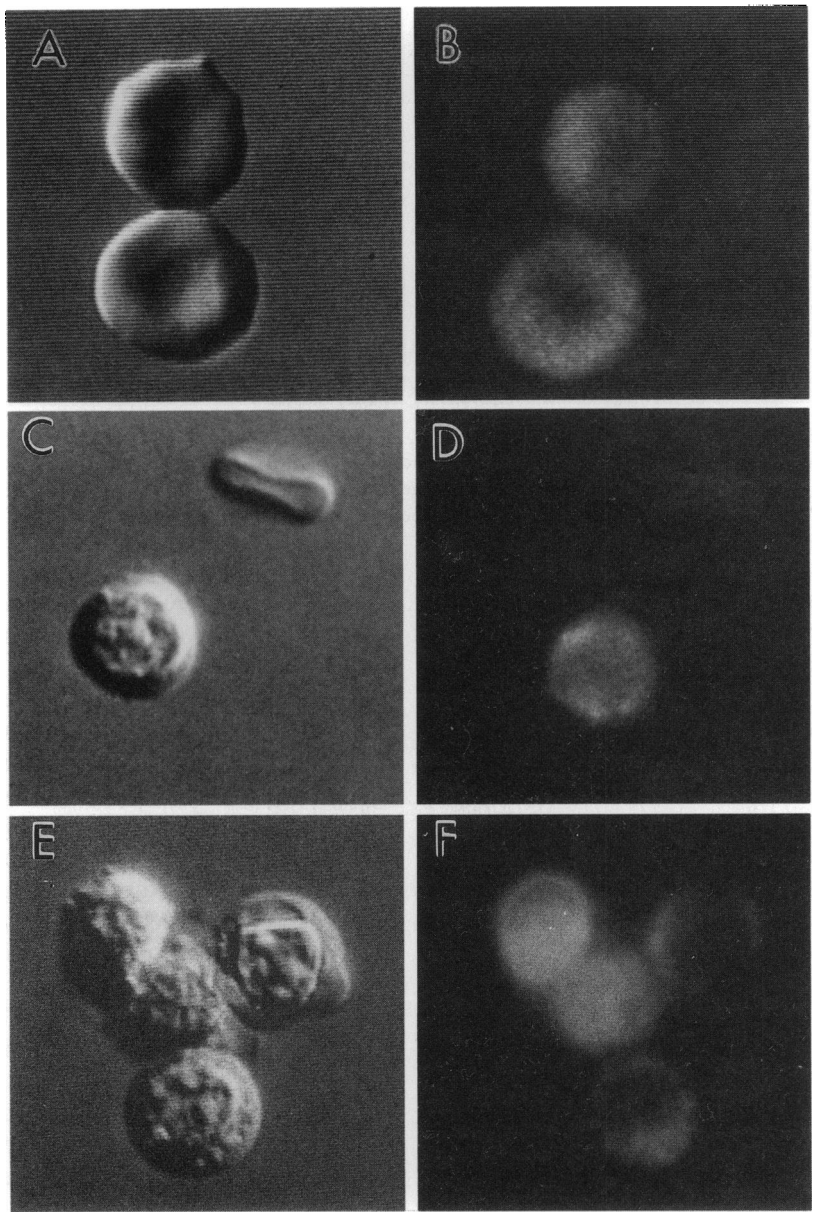

FIGURE 2 Photomicrographs of peripheral blood lymphocytes and erythrocytes stained with DCs. (*A*, *C*, and *E*) Differential interference contrast images; and (*B*, *D*, and *F*) fluorescence micrographs of cells stained with DCs. Erythrocytes exhibit a uniform DCs staining pattern (*A* and *B*). In *B* the camera gain was increased to detect the weak DCs staining seen in erythrocytes. PBLs exhibit bright, diffuse, punctate, and dim DCs-staining patterns (*D* and *F*). *D* shows that lymphocytes label much more brightly with DCs than do erythrocytes. Cells were incubated with 3×10^{-6} M DCs for 2–4 h at 37°C and washed twice with PBS before analysis. (×1,000)

relevant concentrations (i.e., 1–5 μM) that were below its critical micelle concentration. We suggest that the two-component fit is a reflection of an interaction between DCs and cells.

Time-resolved fluorescence anisotropy

TRFA is sensitive to the rotational motions of a fluorophore occurring near the time scale as its fluorescence lifetime (18, 19). Fig. 5 is a representative TRFA plot of peripheral blood lymphocytes labeled with DCs. The time-dependent decay is a measure of the rotational correlation time (3.0 ± 0.1 ns) that reflects the probe's rotational mobility. The time-dependent component of the TRFA is the limiting anisotropy (r_∞ = 0.08); it corresponds to a dansyl cone angle that apparently is 51.5° (calculated using Eq. 3) (17, 19). The limiting anisotropy of DCs suggests that it wobbles more than DPH (18) in leukocyte membranes (this would still be true if DCs were immobilized at a second site since r_∞ would be overestimated). These anisotropy studies indicate that the rotation of DCs is hindered at one or more

TABLE 1 **Properties of DCs in various solvents and cells**

Solvent/sample (n)	ϵ	Emission maxima	Fluorescence lifetime	DW	χ^2
		nm	*ns*		
Chloroform (5)	4.8	491	14.5 ± 0.4	1.91	1.05
Acetone (5)	20.7	509	13.1 ± 0.2	2.00	1.08
Ethanol (5)	24.3	515	13.9 ± 0.1	1.82	1.14
Methanol (5)	32.6	522	13.0 ± 0.1	2.09	1.09
H_2O/HBSS (8)	80.4	522	17.6 ± 2.1	1.92	1.06
Erythrocytes (3)	NA	490	***13.2 ± 2.4	1.35	1.70
			τ_1 = 1.3 ± 0.3	2.11	0.88
			τ_2 = 13.9 ± 1.0		
Peripheral blood lymphocytes (9)	NA	484	***16.9 ± 1.2	1.02	2.09
			τ_1 = 4.7 ± 1.0	2.01	1.01
			τ_2 = 18.5 ± 1.0		

Emission spectra and fluorescence lifetimes of DCs were measured in various solvents and test samples. Five × 10^{-6} M DCs was added to the samples at 37°C for 1 h. Cell samples were washed twice with PBS (pH 7.4) and analyzed at 37°C. Emission spectra (10-nm slit width) were obtained with an excitation wavelength of 360 nm (5-nm slit width). Fluorescence lifetime decay measurements used the nitrogen-337 nm peak for excitation. Terms used: n, number of independent runs; ϵ, dielectric constant; DW, Durbin-Watson test parameter (should be >1.65 and >1.75 for the fluorescence decay curve fit of single and double exponentials, respectively); NA, not applicable; and χ^2, chi-square determined for the same fluorescence decay curve fit. Asterisks (***) refer to the best single exponential fit of the decay curve in the cell samples. Above data are means of all sample runs.

site(s) of localization within lymphocytes. These results indicate that a binding site(s) of DCs may be anisotropic in human PBLs.

DISCUSSION

In this study we have used fluorescence techniques to characterize the binding of the immunosuppressive drug DCs to lymphocytes. Four specific fluorescence techniques were used to examine the nature of DCs-binding to PBLs. (*a*) Video-intensified fluorescence microscopy of DCs showed uniform labeling of lipid vesicles and erythrocytes, whereas lymphocytes exhibited heterogeneous staining patterns in PBLs. (*b*) Fluorescence emission maxima of DCs in various solvents tested the sensitivity of DCs to hydrophobic environments and then the DCs emission maxima in erythrocytes and PBLs could then be characterized as hydrophobic. (*c*) DCs fluorescence lifetimes in solvents and cells were analyzed, and the presence of two distinct lifetime components suggest that DCs binds to a heterogeneous site or multiple sites with different local environments in PBLs. (*d*) Fluorescence anisotropy of DCs illustrates that at least one component of the cyclosporine label moves in an anisotropic fashion in PBLs.

Fluorescence micrographs of DCs in lymphocytes revealed many different patterns of staining from uniform plasma membrane staining to punctate staining (cytosolic binding sites) and combinations of both. It was apparent from the micrographs that lymphocytes contained binding sites at the plasma membrane and in the cytosol. In contrast, micrographs of labeled erythrocytes and lipid vesicles exhibited only a uniform diffuse staining, possibly reflecting the exclusive binding of DCs to these membranes.

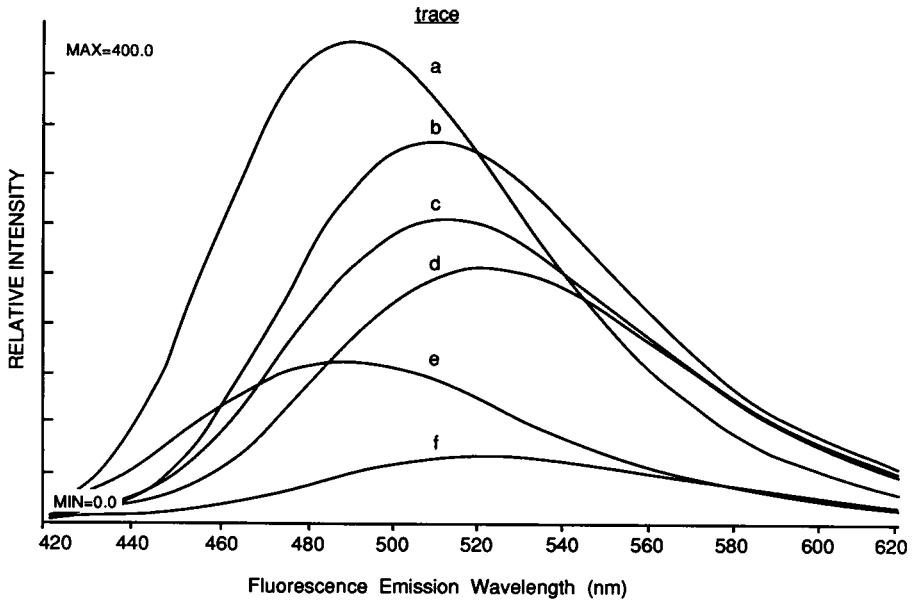

FIGURE 3 DCs emission spectra in various solvents and cells. Five × 10^{-6} M DCs was added to the appropriate solvent and allowed to equilibrate for 1 h at 37°C. The solvents shown are chloroform (trace *a*), acetone (*b*), ethanol (*c*), methanol (*d*), and aqueous (*f*). PBLs are shown in trace *e*. Cells were stained similarly but washed twice with PBS (pH 7.4) before analysis. Aqueous buffer samples were prepared with 0.1% Triton-X to minimize the effects of aggregates.

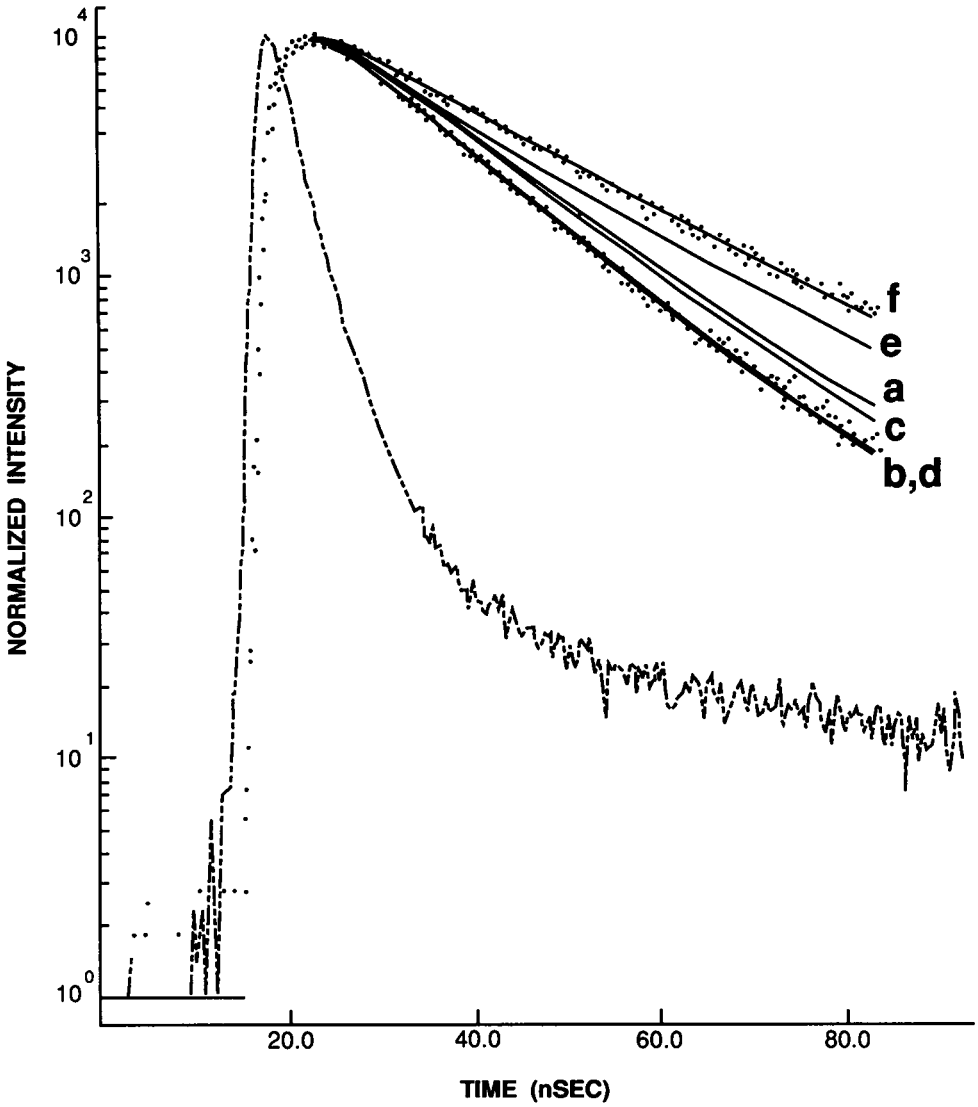

FIGURE 4 Fluorescence lifetime decay curves of DCs in solvents and cells. The dashed line (---) is the background lamp curve. The solid lines (——) are overlapped decays of DCs in the following samples: (*a*) chloroform, (*b*) acetone, (*c*) ethanol, (*d*) methanol, (*e*) H_2O/HBSS, and (*f*) peripheral blood lymphocytes. Data points (· · ·) are included only for the upper- and lower-most decay curves. The first 18 ns were reserved for background counts. PBLs exhibit a unique heterogeneous DCs lifetime when compared with all other samples, including erythrocytes.

Both fluorescence emission spectra and fluorescence lifetimes of DCs are dependent on the hydrophobicity of it's environment. The emission maxima of DCs in chloroform, erythrocytes, and lymphocytes are all similar (480–490 nm), indicating hydrophobic environments (an aqueous environment yields a DCs emission maximum = 522 nm). Comparing measurements in standard solvent systems with those cells showed that DCs is primarily localized in nonpolar environments within erythrocytes and lymphocytes.

The monoexponential decay of DCs fluorescence in all the solvents tested was expected of these homogeneous systems. In erythrocytes, DCs exhibited an emission maximum similar to that found for nonpolar solvents, suggesting that DCs is localized in a hydrophobic environment, apparently the lipid bilayer. The fluorescence lifetime decay of DCs in erythrocytes required two exponents for a reliable fit. Peripheral blood lymphocytes labeled with DCs resulted in a complex lifetime curve that required two exponentials. But these two exponentials were different from those measured for DCs in erythrocytes. In erythrocytes, $\tau_1 = 1.3 \pm 0.2$ and $\tau_2 = 13.9 \pm 0.9$ ns, whereas DCs in PBLs gave $\tau_1 = 4.7 \pm 1.0$ and $\tau_2 = 18.5 \pm 1.0$ ns. If only bilayer partitioning of DCs were involved, one would not expect such large differences between the fluorescence lifetime decay of DCs. Furthermore, the short lifetime component could not be observed in DMPC vesicles. These results support the conclusion that the lymphocyte has DCs binding sites that are physically, and perhaps physiologically, differ-

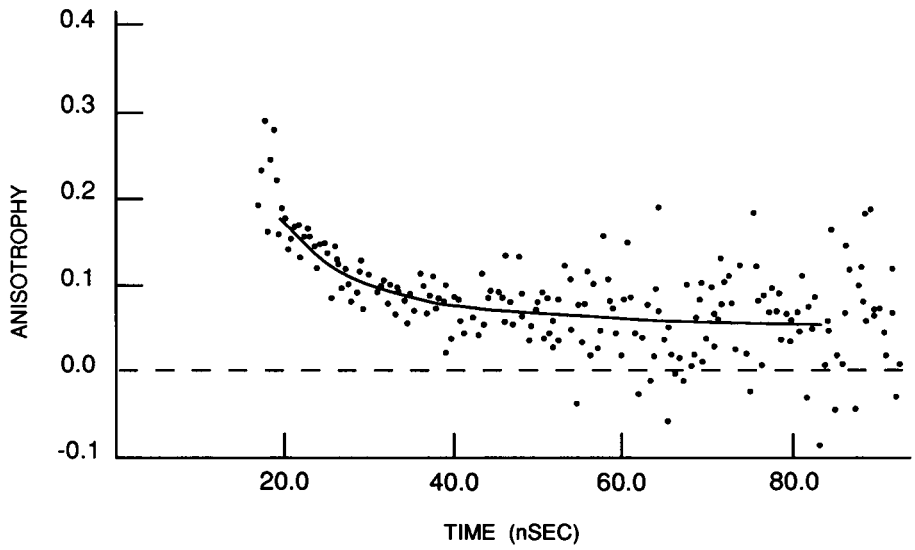

FIGURE 5 TRFA of DCs in PBLs. Cells were incubated with 5×10^{-6} M DCs for 1 h at 37°C and washed twice with PBS (pH 7.2) before analysis. The raw data points (· · ·) and the least-squares fit (——) are shown. The first 9 ns were reserved for background counts. This representative TRFA plot provides direct evidence that DCs is localized in a heterogeneous and anisotropic environment when bound to PBLs (n = 3).

ent from those found in erythrocytes. For example, the short lifetime component in cells may correspond to a cytosolic binding protein site (13, 14). TRFA plots confirm that a population of DCs molecules is indeed bound to some site(s) in the lymphocyte and is not unattached.

Another possible interpretation of the data is that different subsets of lymphocytes are binding DCs in different manners. Indeed, Hess and Colombani (14) have shown differential DCs staining among T lymphocytes; some cells are dim, whereas others are brightly labeled. Other evidence presented here is also consistent with the interpretation that more than one binding site may be present on an individual lymphocyte. Fluorescence micrographs of DCs-labeled peripheral blood lymphocytes show most cells labeling in a heterogeneous manner (diffuse and punctate staining). This is consistent with other findings that CsA partitions into the lipid bilayer (11, 22) and also that CsA binds a cytosolic protein (13, 14). The heterogeneous DCs binding sites that are found in PBLs may reflect the physiologically relevant site(s) of CsA action. Recent advances in streak camera technology may make it possible to image microscopically variations in fluorescence lifetimes on a cell-by-cell basis and within compartments of an individual cell in the near future.

Various studies indicate that CsA alters lymphocyte physiology by interacting with cytosolic proteins and perturbing membrane functions. A depolarization of the lymphocyte plasma membrane occurs 2–5 min after CsA exposure (10) and causes lipid ordering in lymphocyte membranes during the same time frame (8), as well as alterations in phospholipid metabolism (6). Evidence presented here of heterogeneous cyclosporine binding sites in human PBLs is consistent with the findings of others that cyclosporine may partition into membranes (22), forms cytosolic lipid droplets (16), and binds a cytosolic protein in target cells (13, 14). Our results suggest that cyclosporine binds to a heterogeneous site and/or multiple physically distinct sites in living lymphocytes.

We thank Drs. R. Wenger, D. Romer, and E. Rissi at Sandoz Ltd. (Basel, Switzerland) for their generous gift of the (n-dansyl)-o-lysyl-8-cyclosporine used in this study.

This work has been supported by National Institutes of Health grant AI-27409. It is also submitted as partial fulfillment of the degree of Doctor of Philosophy by C. D. Niebylski.

Received for publication 20 August 1992 and in final form 30 October 1992.

REFERENCES

1. Borel, J. F. 1976. Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity. *Immunology.* 31:631–639.
2. Deeg, H. J., R. Storb, P. L. Weiden, R. F. Raff, G. E. Sale, K. Atkinson, T. C. Graham, and E. D. Thomas. 1982. Cyclosporin A and methotrexate in canine marrow transplantation: engraftment, graft-versus-host disease, and induction of intolerance. *Transplant.* 34:30–35.
3. Kupiec-Weglinski, J. W., P. A. Lear, C. D. Heidecke, D. Araneda, and N. L. Tilney. 1984. Restoration of allograft responsiveness in B rats. IV. The divergent migratory behavior of lymphocyte populations mediating cardiac allograft rejection. *Cell. Immunol.* 85:459–476.
4. Bunjes, D., J. Hardt, M. Rollinghoff, and H. Wagner. 1981. Cyclosporin A mediates immunosuppression of primary cytotoxic T cell responses by impairing the release of interleukin 1 and interleukin 2. *Eur. J. Immunol.* 11:657–661.
5. Larsson, E. L. 1980. Cyclosporin A and dexamethasone suppress T cell responses by selectively acting at distinct sites of the triggering process. *J. Immunol.* 124:2828–2833.

6. Szamel, M., P. Berger, and K. Resch. 1986. Inhibition of T lymphocyte activation by cyclosporine A: interference with the early activation of plasma membrane phospholipid metabolism. *J. Immunol.* 136:264–269.
7. Shevach, E. M. 1985. The effects of cyclosporine A on the immune system. *Annu. Rev. Immunol.* 3:397–423.
8. Niebylski, C. D., and H. R. Petty. 1990. Cyclosporine A induces an early and transient rigidification of lymphocyte membranes. *J. Leukocyte Biol.* 49:407–415.
9. Damjanovich, S., A. Aszalos, S. Mulhern, M. Balazs, and L. Matyus. 1986. Cytoplasmic membrane potential of mouse lymphocytes is decreased by cyclosporins. *Mol. Immunol.* 23:175–180.
10. Gelfand, E. W., R. K. Cheung, and G. B. Mills. 1986. The cyclosporines inhibit lymphocyte activation at more than one site. *J. Immunol.* 138:1115–1120.
11. Niwa, Y., T. Kano, S. Taniguchi, Y. Miyachi, and T. Sakane. 1986. Effect of cyclosporine A on the membrane-associated events in human leukocytes with special reference to the similarity with dexamethasone. *Biochem. Pharmacol.* 35:947–951.
12. O'Leary, T. J., P. D. Ross, M. R. Lieber, and I. W. Levin. 1986. Effects of cyclosporine A on biomembranes: vibrational spectroscopic, calorimetric and hemolysis studies. *Biophys. J.* 49:795–801.
13. Colombani, P. M., A. Robb, and A. D. Hess. 1985. Cyclosporin A binding to calmodulin: a possible site of action on T-lymphocytes. *Science (Wash. DC).* 228:337–339.
14. Hess, A. D., and P. M. Colombani. 1986. Mechanism of action of cyclosporine: role of calmodulin, cyclophilin, and other cyclosporine-binding proteins. *Transplant. Proc.* 18:219–237.
15. Ryffel, B., U. Gotz, and B. Heuberger. 1982. Cyclosporin receptors on human lymphocytes. *J. Immunol.* 129:1978–1982.
16. Koponen, M., and F. Loor. 1983. Cytoplasmic lipid droplets as the possible eventual cellular fate of active forms of cyclosporin. *Exp. Cell Res.* 149:499–512.
17. Lakowicz, J. R. 1980. Principles of Fluorescence Spectroscopy. Plenum Press, New York. 496 pp.
18. Petty, H. R., C. D. Niebylski, and J. W. Francis. 1987. Influence of immune complexes on macrophage membrane fluidity: a nanosecond fluorescence anisotropy study. *Biochemistry.* 26:6340–6340.
19. Kinosita, K., Jr., R. Kataoka, Y. Kimura, O. Gotoh, and A. Ikegami. 1981. Dynamic structure of biological membranes as probed by 1,6-Diphenyl-1,3,5-hexatriene: a nanosecond fluorescence depolarization study. *Biochemistry.* 20:4270–4277.
20. Weaston, H. P., editor. 1970. Handbook of Chemistry and Physics. 51st ed. The Chemical Rubber Co., Cleveland, OH. 2063 pp.
21. Grinvald, A., and I. Z. Steinberg. 1974. On the analysis of fluorescence decay kinetics by the method of least squares. *Anal. Biochem.* 59:583–598.
22. LeGrue, S. J., A. W. Friedman, and B. D. Kahan. 1983. Binding of cyclosporine of human lymphocytes and phospholipid vesicles. *J. Immunol.* 131:712–718.