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KINETICS OF UPTAKE AND INTRACELLULAR BINDING OF CYCLOSPORINE A IN RAJI CELLS, *IN VITRO**

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Abstract—Uptake characteristics of Cyclosporine A (CsA), an immunosuppressive agent widely used in organ transplantation, have been evaluated in RAJI cells, a human Burkitt lymphoma cell line which (i) does not bear T-cell markers, (ii) is insensitive to CsA after a 1 hr exposure to concentrations up to 50 μg/ml, and (iii) does not metabolize CsA. CsA is rapidly accumulated inside the cells until a near steady-state is achieved (within 1–3 min). This uptake is characterized by two components: one linear process saturable at low drug concentrations (lower than 1 μg/ml) and another not saturable component even at high drug concentrations (up to 50 μg/ml). Uptake of CsA is temperature-dependent and unaffected by the presence of CsD, a structural CsA analog (50 μg/ml CsD) or sodium azide (10 mM) in the extracellular compartment.

Intracellular accumulation of CsA is associated with the rapid appearance of a cytosolic drug–protein complex, which is responsible at least in part, for the large amount of total drug accumulated inside the cells. Chromatographic analysis of this (^{3}H)CsA-macromolecule complex on a Bio-Gel P-60 exclusion column demonstrates that the molecular weight of this protein(s), likely cyclophilin, is around 15,000–20,000 daltons. Using Lineweaver–Burk analysis of binding equilibrium data, the dissociation constant of CsA for this binding site was approximately 2.2 μM.

These studies, which demonstrate that CsA (i) is rapidly accumulated inside the cells as free drug but is also specifically bound to an intracellular macromolecule, and (ii) is selectively retained in the intracellular compartment after the extracellular drug is removed, could explain the intense distribution of CsA in the organs and the slow disappearance of CsA from plasma after CsA therapy in humans.

Cyclosporine A§, a new potent immunosuppressive agent [1], is widely used in human organ transplantation [2, 3]. Other applications of CsA in autoimmune diseases [4] and schistosomiasis [5] have also been reported.

CsA seems to act on the immune system by inhibiting the initial steps of T-lymphocyte activation [6]. Other studies have investigated the possible effects of CsA on the production of growth factors like IL 1 and IL 2 [7–9].

The immunosuppressive activity of this molecule is starting to be understood but little is known about the drug uptake and its intracellular distribution. Ryffel *et al.* [10, 11] reported the different accumulation of CsC§, another biologically active cyclosporine [10], by murine and human immune cells and its specific binding on lymphocyte sites closely associated with the mitogenic receptor [11]. Merker *et al.* [12, 13] showed that uptake and concentration of (^{3}H)CsA in thymoma cell line (BW 5147) happened mainly in the cytosol and not in the cell membrane. The protein responsible for the specific concentration of the immunosuppressant CsA in lymphoïd cells, namely cyclophilin, has an apparent molecular weight of 15,000–20,000 daltons [14]. Additional studies have demonstrated its presence in many malignant and normal cells [15]. The highly conserved structure and broad distribution of this protein suggest that cyclophilin and a ligand(s) may play a key role in cell growth and differentiation [15].

In this study we report (i) the kinetic of CsA uptake, and (ii) the rates of association of CsA with an intracellular binding site(s) using a Burkitt lymphoma cell line as a model. Although CsA was known to have no biological activity on this kind of cell line, it was appropriate to compare the intracellular disposition of CsA in CsA-sensitive cell lines, T-lymphocytes or thymic lymphoma [13], and in a CsA-insensitive cell line, Burkitt lymphoma cells.

MATERIALS AND METHODS

Materials. Tritiated CsA (15.5 mCi/mg) was obtained from Sandoz Ltd (Basle, Switzerland) and was 99% pure by HPLC; this compound was used without further purification. Standards CsA and CsD were provided in powder form and dissolved initially at room temperature in absolute ethyl alcohol. Subsequent dilutions were performed using

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§ Abbreviations used: CsA, Cyclosporine A; CsC, Cyclosporine C; CsD Cyclosporine D; HPLC, high performance liquid chromatography.

RPMI-1640 medium and stored at −20° without apparent degradation over a month.

Bio-Gel P-6 (200–400 mesh) and Bio-Gel P-60 (100–200 mesh) were purchased from Bio-Rad Laboratories (Richmond, CA). The following molecular weight markers, egg ovalbumine (M_r 45,000), carbonic anhydrase (M_r 29,000), cytochrome *c* (M_r 12,384) and vitamin B_{12} (M_r 1355), were obtained from commercial sources.

Cells. The human Burkitt lymphoma cell line, RAJI, was propagated in continuous culture in Roswell Park Memorial Institute medium (RPMI) 1640 (Biomerieux) supplemented with 15% heat-inactivated undialyzed fetal calf serum (Flobio-Gibco) and 2 mM L-glutamine. For cell growth experiments, cells at a final concentration of $5\ 10^5$ cells per ml of RPMI medium, were exposed for 1 hr to NaCl buffer (control) or increasing CsA concentrations. Cells were washed twice then resuspended in RPMI 1640 medium supplemented with fetal calf serum and 2 mM L-glutamine. Cells were scored after 48 hr of incubation at 37° in a 5% CO_2 humidified atmosphere.

Cell growth was defined as follows:

$$\text{Percentage of growth} = \frac{\text{no of cells in drug-treated cultures}}{\text{no of cells in control cultures}} \times 100$$

Preparation of cells for analysis of intracellular radiolabel. Exponentially growing cells were harvested, washed twice with ice-cold 0.85% NaCl solution and then resuspended in RPMI medium. CsA was then added to the cell suspension. The pH was maintained at 7.4 by passing warm and humidified mixture of 95% O_2:5% CO_2 and the cell suspension was stirred with a Teflon paddle in flasks immersed in a 37° water bath [16].

CsA uptake was estimated after treatment of the cells with (^{3}H)CsA (specific activity: 500–1000 cpm/ng). Transport fluxes were stopped by adding 10 vol. of ice-cold 0.85% NaCl solution and the cells were washed twice using the same solution. The cell pellet was then removed by a Pasteur pipet, transfered on a polyethylene tare, and dried overnight at 70°. The dry pellets were weighted, placed in scintillation vials, and incubated in 0.2 ml of 1 N KOH for 1 hr at 70°. The digested solution was then neutralized with 0.2 ml of 1 N HCl. Five millilitres of Ready Solv scintillation cocktail (Beckman Instruments) were then added and the radioactivity evaluated. Results are expressed as nanomoles of equivalent CsA accumulated per gram of dry weight, as previously described in detail [16].

Intracellular radioactivity was further investigated by a reversed-phase HPLC method. Cell extract was boiled for 2 min and centrifuged. Supernatant fraction was analyzed on a Hewlett Packard 1090 A gradient liquid chromatograph equipped with a 10 μm C_{18} μBondapak column (Waters Associated). The HPLC analysis consisted of an isocratic elution for 14 min with 42% bidistilled water and 58% acetonitrile, followed by a gradient from 58 to 70% of acetonitrile over 20 min [17].

Binding to intracellular macromolecules. Cells exposed to radiolabeled CsA were washed twice as described above. Cell pellet was resuspended in ice-cold Tris buffer (20 mM, pH 7.2) containing 2-mercaptoethanol (5 mM) and sodium azide (0.02%) (buffer A; 14). The cells were disrupted by sonic oscillation until over 95% of them were broken as established by light microscopy and the cellular debris were removed by centrifugation at 40,000 *g* for 20 min. A 1 ml supernatant sample was then analyzed on a mini-column (5 ml) of Bio-Gel P-6, pre-equilibrated in buffer A by rapid centrifugation according to the method described by Fry *et al.* [18]. This procedure can separate the protein–ligand complex from the free ligand which is completely retained within the column. This was further confirmed by adding radiolabeled CsA on the mini-column; the latter was quantitatively retained inside the gel.

Values are the mean ± SD of at least three different experiments.

In some cases, the intracellular protein-CsA complex was further chromatographed on a Bio-Gel P-60 column (40 cm × 1.6 cm) equilibrated with buffer A and calibrated with the following molecular weight markers; ovalbumine, carbonic anhydrase, cytochrome *c* and vitamin B_{12} respectively.

RESULTS

Cell growth experiments

RAJI cells were incubated for 48 hr in a 5% CO_2-humidified atmosphere after a 1 hr exposure to increasing CsA concentrations ranging from 0.1 to 25 μg/ml. Over this range of concentration cell growth was not perturbated. At 25 μg/ml, cell growth was approximately 85% compared to control cells.

Uptake of CsA in RAJI cells

After exposure of RAJI cells to CsA, the drug is rapidly accumulated inside the cells. The initial uptake is studied over a short period ranging between few seconds (4–7 sec) to 90 sec. Moreover and based upon previously reported data [13] showing the specific intracellular binding of CsA in a murine

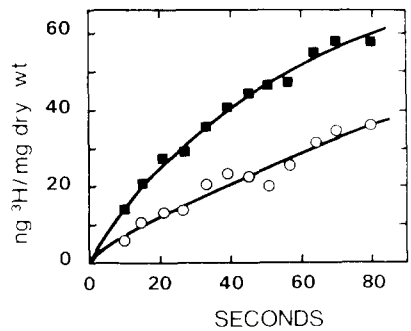

Fig. 1. Time-courses of uptake and intracellular binding of CsA, 10–80 sec after exposure of cells to 500 ng/ml (^{3}H)CsA. At specified times, portions of cells were separated, washed twice at 0°, and both total (^{3}H) uptake (■—■) and (^{3}H) associated with intracellular macromolecules (○—○) were monitored as described in Materials and Methods.

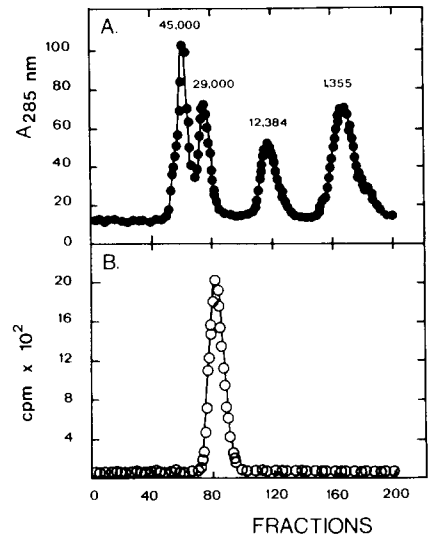

Fig. 2. Elution profile of the CsA–protein complex on Bio-Gel P-60. (A) Elution of the molecular weight markers. (B) Chromatogram of the CsA–protein complex after incubation of RAJI cells with 500 ng/ml (^{3}H)CsA for 30 min.

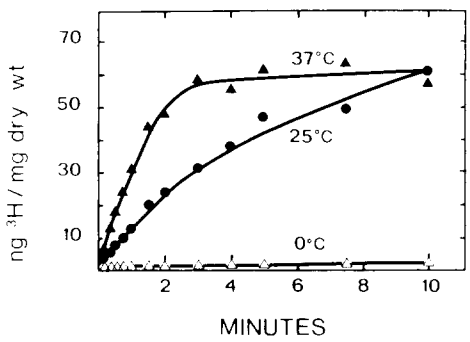

Fig. 3. Effect of temperature on CsA uptake and accumulation in RAJI cells. Cells were exposed to 500 ng/ml (^{3}H)CsA in RPMI medium at different temperatures: 37° (▲—▲), 25° (●—●) and 0° (△—△). At specified times, portions of cells were separated, washed twice and total (^{3}H) analyzed as described in Materials and Methods.

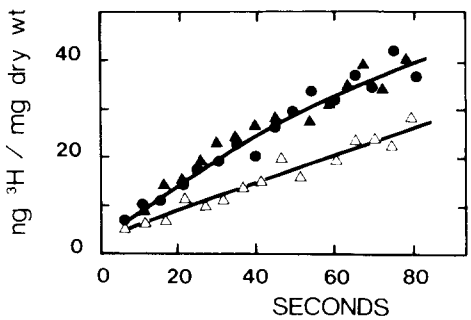

Fig. 4. Time-courses of CsA uptake in control cells or in CsA-preloaded cells. Cells were exposed for 30 min in drug-free medium (●—●) or in medium containing 500 ng/ml (▲—▲) or 10 μg/ml (△—△) unlabeled CsA. Cells were washed twice then resuspended in fresh medium containing 500 ng/ml (^{3}H)CsA and uptake was monitored as described in Materials and Methods.

thymoma cell line, the kinetic of binding of CsA to intracellular macromolecule(s) is also investigated.

RAJI cells are incubated with 500 ng/ml (^{3}H)CsA and, at specified times, intracellular content is analyzed for total radioactivity and for binding to macromolecules. Results are illustrated in Fig. 1. Accumulation of CsA is rapid, non-linear and specifically associated to intracellular macromolecules. Over this time-period, approximately 40% of the intracellular constituents are bound to macromolecules. To characterize further this intracellular binding site, cells pre-exposed to radiolabeled CsA are disrupted by sonic oscillation and cellular debris removed by centrifugation. Protein–ligand complex(es) obtained after Bio-Gel P-6 chromatography is then applied on a Bio-Gel P-60 column calibrated with appropriate molecular weight markers. As illustrated in Fig. 2, CsA is bound to a protein(s) with a molecular weight around 20,000 daltons.

Different characteristics of CsA uptake

The uptake of CsA is estimated after exposure of RAJI cells to 500 ng/ml (^{3}H)CsA. At 37°, cells rapidly accumulate CsA and an intracellular concentration of 58.3 ∓ 16.5 ng/mg of cell dry weight (N = 7) is reached within 10 min and is constant for at least 1 hr. Assuming that intracellular water/dry weight ratio is approximately 2 μl/mg of dry weight, this represents a 500-fold increase over the drug concentration in the medium. At 25° the initial uptake of drug is slower but, after 10 min, the same final intracellular concentration is achieved (Fig. 3). At 0° the cells do not accumulate appreciable amounts of drug even after 30 min of incubation. The low radioactive background can be attributed to a specific and/or a non specific binding on the extracellular membrane.

The energy-dependence of CsA transport and accumulation in cells has been further studied using sodium azide, a metabolic poison. The initial uptake of 500 ng/ml (^{3}H)CsA as well as total accumulated drug, are not affected by the presence of 10 mM sodium azide in the extracellular medium. Total drug accumulated after 60 sec was 45.9 ∓ 14.5 ng/mg dry weight (N = 2) in control cells and 50.6 ∓ 20.3 ng/mg dry weight in sodium azide-treated cells.

Moreover, the presence of 50 μg/ml CsD, a structural CsA analog, in the extracellular medium has no effect on the initial rate of uptake of 500 ng/ml (^{3}H)CsA. Total drug accumulated after 60 seconds was 45.9 ∓ 14.5 ng/mg dry weight (N = 2) in control cells and 47.0 ∓ 11.3 ng/mg dry weight in CsD-treated cells.

The rates of uptake of CsA in control cells and in CsA-pretreated cells are also determined (Fig. 4). Cells are exposed for 30 min in drug-free medium or in medium containing 500 ng/ml or 10 μg/ml unlabeled CsA. Cells are washed twice at 0° to eliminate extracellular drug, then resuspended in 37° fresh medium containing 500 ng/ml (^{3}H)CsA. The rates of uptake are determined over 80 sec. In 500 ng/ml CsA-preloaded cells, uptake of radiolabeled CsA is identical to that of the control. However, in 10 μg/ml CsA-preloaded cells, conditions under which the intracellular binding site(s) is saturated with un-

labeled CsA (see below), uptake of radiolabeled CsA was only 60% of that of control. These results suggest an important role for the intracellular binding site(s) in the uptake of CsA.

Preliminary results from our laboratory demonstrate that CsA can be extensively metabolized in some normal cells, e.g. freshly isolated rabbit hepatocytes [17]. So, the behavior of (^{3}H)CsA in RAJI cells is examined using a new specific HPLC method which permits to resolve CsA from its major metabolite forms [17]. After a 1 hr exposure of RAJI cells to 500 ng/ml (^{3}H)CsA (specific activity = 3380 cpm/ng), 99% of the intracellular drug was recovered as unchanged CsA.

Time-dependence of accumulation and intracellular binding of CsA

The time courses of total intracellular accumulation (upper panel) and binding (lower panel) of CsA after exposure of cells to increasing concentrations of (^{3}H)CsA, 100, 500 and 1000 ng/ml, are illustrated in Fig. 5.

For each concentration studied, the cells rapidly accumulate CsA until they reached a plateau (within 10 min). This steady-state value remains constant over the following 20 min of observation (upper panel). The kinetics of intracellular binding of CsA are shown, for the same CsA extracellular concentrations, in the lower panel of Fig. 5. The rates of fixation of CsA to the intracellular site(s) are rapid over the first 5 min of observation, then reach a maximum value when total accumulation in cells has achieved a steady-state.

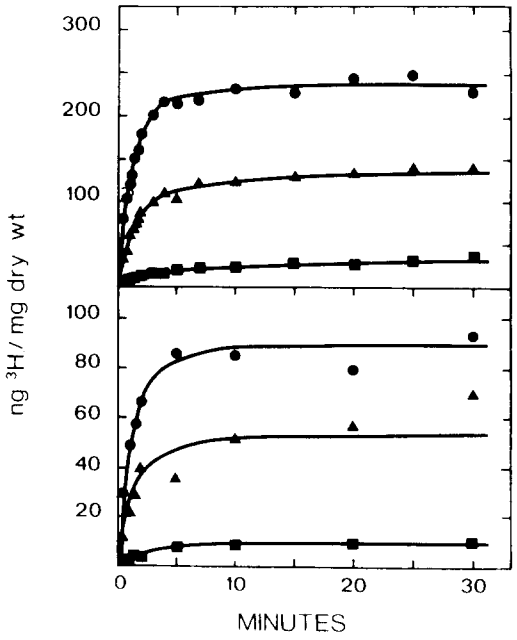

Fig. 5. Time-dependence of intracellular accumulation and binding of CsA in RAJI cells. Cells were exposed to increasing concentrations of (^{3}H)CsA: 100 ng/ml (■—■), 500 ng/ml (▲—▲) and 1000 ng/ml (●—●). Total drug accumulation (upper panel) and intracellular binding (lower panel) were monitored as described in Materials and Methods.

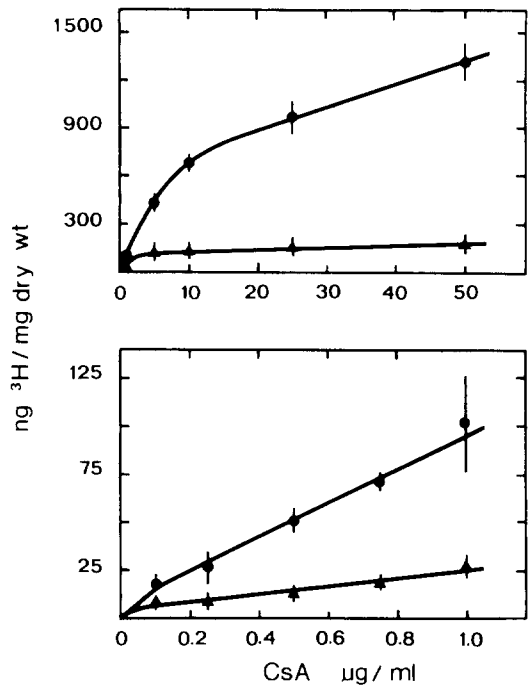

Fig. 6. Concentration-dependence of intracellular accumulation and binding of CsA in RAJI cells. Cells were exposed for 10 min to increasing concentration of (^{3}H)CsA and both total (^{3}H) uptake (●—●) and intracellular binding (▲—▲) were monitored as described in Materials and Methods. Each result is the mean ± SD of two different experiments performed in duplicate.

Concentration-dependence of accumulation and intracellular binding of CsA

In the experiments described above, we showed that after a 5 min exposure to increasing concentrations of radiolabeled CsA, intracellular accumulation and binding have both achieved a steady-state value which persists over the 1-hr period of observation. In the following experiment, cells are exposed for 10 min to increasing concentrations of (^{3}H)CsA and intracellular radiolabel is analyzed for both total radioactivity and specific binding to macromolecules (Fig. 6).

When the extracellular concentration of (^{3}H)CsA varies from 0.1 to 50 µg/ml, uptake of total radiolabel gives evidence of two components: one linear process which saturates at concentrations up to 1–5 µg/ml and another nonsaturating component for concentrations above 10 µg/ml. The lower panel of this figure emphasizes the linear uptake process for concentration ranging between 0.1 and 1 µg/ml.

Specific analyses of radiolabel bound to intracellular macromolecules show that this intracellular binding site(s) is saturable at extracellular CsA concentrations above 10 µg/ml. Indeed, while the ratio "protein–CsA complex/total accumulated drug" was about 0.2–0.3 for extracellular drug concentrations up to 1 µg/ml, this ratio was further decreasing at higher CsA concentrations.

The binding equilibrium data are evaluated for CsA extracellular concentrations ranging between

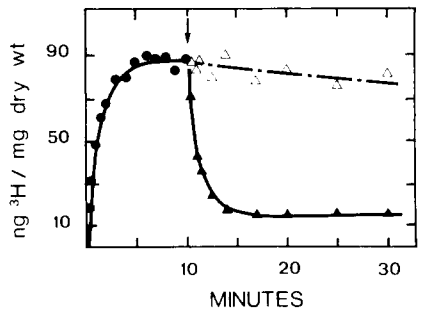

Fig. 7. Intracellular retention of CsA in RAJI cells. Cells were incubated for 10 min with 500 ng/ml (^{3}H)CsA, then separated by centrifugation, washed twice with 0° NaCl, and resuspended in 37° fresh RPMI medium containing no (△—△) or 10 μg/ml (▲—▲) unlabeled CsA at the time indicated by the arrow. Total intracellular (^{3}H) was measured at the selected times as described in Materials and Methods.

0.25 and 50 μg/ml and have been further analyzed using a Lineweaver–Burk plot in order to calculate the dissociation constant for this binding component. Dissociation constant is around 2.2 μM.

Intracellular retention of CsA

Cells are loaded for 10 min with 500 ng/ml (^{3}H)CsA, washed twice with 0° NaCl buffer, and resuspended into fresh RPMI medium at 37° in the absence or presence of unlabeled CsA (10 μg/ml) (Fig. 7).

In the absence of extracellular CsA, there is a slow loss of intracellular radioactivity over 30 min suggesting a selective retention of CsA in the intracellular medium. However, in the presence of extracellular CsA (10 μg/ml), there is a very rapid decrease in intracellular CsA concentration—representing a loss of 70.88% ∓ 6.90% (N = 3)—until a new steady-state is achieved.

DISCUSSION

In this paper we describe the kinetics of uptake and intracellular binding of CsA in RAJI cells, a human Burkitt Lymphoma cell line. This cellular model was chosen because of the homogeneity of the cell population and it permits study under a variety of culture. Moreover this cell line does not bear T-cell markers (N. Tubiana, personal communication) and is not sensitive to inhibition by CsA on the following range of concentration tested: 0.1–25 μg/ml. These results are in agreement with those of others which confirm that greater sensitivity to CsA exists for selected malignant cells that bear some T-cell markers [19, 20]. Moreover, it was appropriate to study the uptake of CsA as well as the nature and amount of the binding component in this CsA-insensitive cell line and compare the results to those obtained by others using CsA-sensitive cell lines, in order to demonstrate if the insensitivity of a cell line could be correlated to drug accumulation or specific intracellular binding.

Ryffel *et al.* [10, 11], using human and murine lymphocytes, reported the specific binding of CsC (another CsA structural analog) on peripheral receptors. Binding of (^{3}H)CsC was saturable, time-dependent, reversible and closely associated with the mitogenic receptor on lymphocytes. Their studies did not explore the binding by the cytosol which apparently represents the major site of CsA accumulation in cells [13].

In BW 5147 cells, a murine lymphoma, Merker and Handschumacher [13] characterized the uptake of radiolabeled CsA and its intracellular disposition. This uptake was characterized by two components; one saturable at low drug concentrations and another not saturable even at high drug concentrations. Moreover, they demonstrated that most of the drug concentrated by the cells was located in the cytosol and bound to a low-molecular weight macromolecule, namely cyclophilin [13, 14]. This intracellular macromolecule bound specifically a series of cyclosporin analogs in proportion to their activity in a mixed lymphocyte reaction [14]. Of significant interest is the occurrence of cyclophilin in nonlymphoid tissues, with a high concentration of this protein present in brain and kidney (organs subject to toxic side effects during CsA therapy in humans) but also in B-cells [15]. However, the relationship between the binding site of CsA and the mechanism of CsA action remains to be established.

The rate of uptake of CsA in RAJI cells was assessed using specific radiolabeled CsA. Uptake and binding in these cells, both reached a plateau within 5 min at 37°. The uptake of (^{3}H)CsA is temperature dependent and unaffected by the presence in the extracellular medium of CsD (50 μg/ml), a CsA structural analog, or sodium azide (10 mM), a metabolic poison. Under these different experimental procedures, the disposition of CsA in RAJI cells was further investigated. Neither degradative compounds nor metabolites were recovered in intra- or extra-cellular compartments.

Uptake of radiolabeled CsA in RAJI cells is characterized by two components; one seems to be saturable at low CsA concentration and is affected by the presence of the intracellular binding component, and another non-saturable at higher drug concentration (above 10 μg/ml).

Intracellular accumulation of CsA is associated with the rapid appearance of a cytosolic drug–protein complex, responsible at least in part, for the large amount of total drug accumulated inside the cells. Indeed, in CsA-preloaded cells (10 μg/ml for 30 min), conditions under which the intracellular binding site(s) is saturated with unlabeled CsA, uptake of (^{3}H)CsA is decreased by approximately 40%. However, when cells are preloaded with 500 ng/ml CsA, conditions under which the intracellular binding site(s) is not saturated, uptake of radiolabeled CsA is comparable to that of control.

The specific binding of CsA with an approximately 20,000 daltons macromolecule(s) was assessed using a Bio-Gel P-60 exclusion column, according to Fry [18] and Handschumacher *et al.* [14] techniques. Although under our experimental procedure (incubation medium, exposure time, chromatographic elution) CsA was bound to a protein(s) with a molecular weight of approximately 20,000 daltons, it is difficult to specify if it was a single protein type.

This question remains under further investigation. Binding of radiolabeled CsA was rapid, saturable at CsA extracellular concentrations above 10 μg/ml, time-dependent and rapidly reversible in the presence of a high unlabeled CsA concentration. Moreover, and based upon binding equilibrium data, the dissociation constant of CsA for this binding site was approximately 2.2 μM. This result is not significantly different from those observed by different authors in cell-free systems and ranged between 0.2 and 1 μM [13, 14].

Of particular interest is the observation that when (^{3}H)CsA-preloaded cells are resuspended in 37° fresh drug-free medium, there is only a very low loss of intracellular radiolabel, due in major part to the specific binding of CsA to intracellular macromolecules, but also to the lipophility of the CsA molecule. These observations could explain the long retention of CsA in different tissues and the long plasma terminal half life values (20–60 hr) observed after CsA administration in human patients [21]. This observation is consistent with the broad distribution of "cyclophilin" in many tumor and normal systems [15].

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