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Increased Cyclosporine Uptake by Cells Pretreated with FK506 and Evidence for Binding of Both Drugs to a Common Intracellular Protein

A. Sanghvi, V.S. Warty, W.F. Diven, S. Todo, and T. Starzl

FK506 is a newly described potent immunosuppressive agent. In vitro immunologic studies have indicated a synergism between FK506 and cyclosporine. Our studies have indicated that the cellular uptake of cyclosporine is enhanced by pretreatment of peripheral blood lymphcoytes for 2 to 22 hours with FK506. Maximum enhancement of cyclosporin uptake was observed in the presence of equimolar concentrations of both drugs. Equivalent enhancement was observed with peripheral blood lymphocytes that were freshly isolated or stimulated with phytohemagglutinin. This observation may offer an explanation for the observed immunologic synergism when both drugs are coadministered. Also, uptake and binding characteristics of FK506 were evaluated in human peripheral blood lymphocytes. The saturating concentration of FK506 in these experiments was found to be approximately 0.5 μM. Scatchard analysis of binding data indicated two binding sites with $K_d = 3.9 \pm 1.8 \times 10^{-9}$M (high affinity) and $K_d = 5.2 \pm 0.8 \times 10^{-6}$M (low affinity). After incubations of cells with cyclosporine and FK506, cells were disrupted by sonication and cell contents were analyzed on a Bio-Gel P-60 molecular sieve column. The elution profile indicated that both drugs migrated with an intracellular protein with an approximate molecular weight of 18,000 to 19,000 dalton. These data imply that the intracellular transport of these drugs may involve similar, if not identical, proteins.

Several studies have indicated that FK506, a novel compound, is a potent immunosuppressive agent. Recent in vitro studies have suggested that FK506 possesses immunosuppressive properties similar to those of cyclosporine A, implying that the mechanisms of action of these two drugs may be similar as well. The immunosuppressive effect of FK506 is several hundredfold greater than that of cyclosporine A.[1,2] As previously demonstrated for cyclosporine A, the inhibitory effect of FK506 is seen in mixed leukocyte culture (MLC) and on secondary proliferation of alloreactive T cells harvested from MLC or propagated from organ transplant biopsies.[3] The published data further show that immunosuppression by FK506 may be mediated through an inhibition of interleukin-2 release,[1-3] again emphasizing the similarities between cyclosporin A and FK506.

AIMS

We attempted to (1) study the kinetics of binding and uptake of FK506 by the human peripheral blood lymphocytes, (2) investigate and characterize the intracellular binding of both drugs, and (3) evaluate CsA uptake by human peripheral blood lymphocytes pretreated with FK506.

MATERIALS AND METHODS

Tritiated CsA (Sandoz Pharmaceuticals, NY) and FK506 (Fujisawa, Japan) were used as ethanolic solutions. Culture media and antibiotics were obtained from GIBCO (Grand Island, NY). Bio-Gel P-6 and P-60 were obtained from Bio-Rad (Richmond, CA). All other reagents and supplies came from Fisher Scientific (Pittsburgh, PA).

Peripheral blood mononuclear cells were isolated as described previously.[4] Monocytes were removed by means of an adhering technique. Lymphocytes were harvested, washed twice with ice-cold saline, and then resuspended in culture medium. Both the CsA and FK506 then were added to the cell suspension, and cells were incubated for 1 hour at 37°C in a himidified mixture of 95% O_2:5% CO_2. Transport fluxes were stopped by adding 10 vol cold saline. Cells were washed twice with same cold saline to remove the excess of both drugs. The cell pellet was resuspended in ice-cold Tris buffer (20 mM, pH 7.2) containing 2-mercaptoethanol and sodium azide.

The cells were disrupted by sonic oscillations, and cell debris was removed by means of centrifugation (40,000 *g* for 15 min). A 1-mL supernatant sample was centrifuged on a mini Bio-Gel P-6 column to separate protein-ligand complex from the free ligand that is completely retained in the column. The intracellular protein-CsA and FK506 complex then was chromatographed on a Bio-Gel P-60 column (precalibrated with the appropriate molecular weight protein markers), and the fractions were used to determine CsA and FK506 for protein contents.

Lymphocytes were incubated with different concentrations of FK506 for 1 hour at 37°C. Cells were washed three times with cold saline and incubated in 0.2 mL in KOH for 1 hour at 70°C. The digested solution was used for the measurement of FK506 by means of enzyme immunoassay and protein concentration.

In another set of experiments, cells were incubated in 1% BSA-RPMI 1640 medium in the presence of 1 μg/mL FK506 under culture conditions. Cells were washed and exposed to different concentrations of CsA and FK506 for 2

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hours. Uptake of CsA by the cells under these conditions was evaluated by a measure of radioactivity.

RESULTS

The concentration dependence of intracellular accumulation and binding of FK506 by human peripheral blood lymphocytes were studied. Cells were incubated in different concentrations of FK506 for 1 hour at 37°C. The uptake of FK506 by PBL was seen as a saturable process, with saturation occurring at approximately 0.5 μM concentration of the drug (Fig 1). Scatchard analysis of the binding data was consistent with two distinct classes of binding sites, one with K_d = 3.9 ± 1.8 × 10^{-8}M for the high-affinity sites and the second with K_d = 5.2 ± 0.8 × 10^{-6}M for the low-affinity sites. This analysis further revealed that there are 5.6 ± 1.0 × 10^4 and 2.5 ± 0.9 × 10^6 high- and low-affinity binding sites, respectively, per cell.

In another set of experiments, peripheral blood lymphocytes (100 × 10^6) were incubated with 2 μg FK506 and 1.5 μg CsA in culture medium for 1 hour. Intracellular contents were analyzed for binding of both drugs to macromolecules. Intracellular accumulation of both drugs was rapid, and both appeared specifically associated with intracellular macromolecule(s). Figure 2 illustrates the elution profile of both cyclosporin and FK506 on a calibrated Bio Gel P-60 column. The calibration curve of the molecular-weight markers—ovalbumin, carbonic anhydrase, cytochrome C, and vitamin

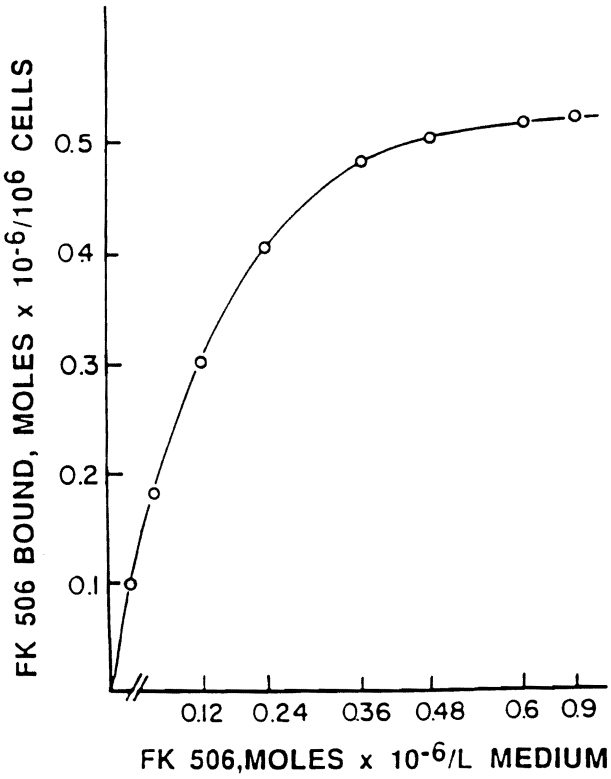

Fig 1.

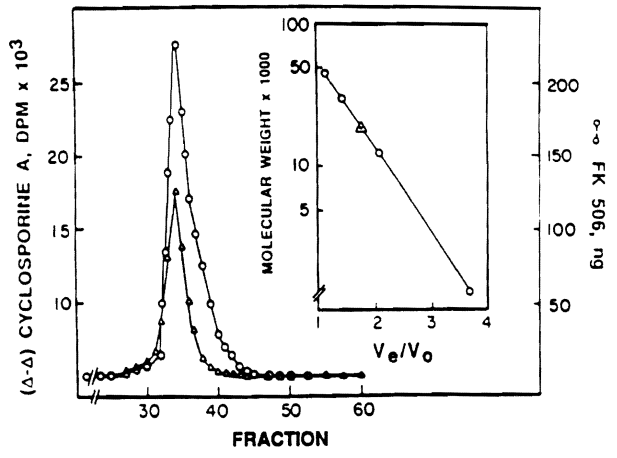

Fig 2.

B^{12}—is shown as an inset. These data show that both cyclosporine A (Δ) and FK506 (●) elute association with protein or proteins with a molecular weight of about 18,000–19,000 dalton.

Figure 3 shows the effects of including equivalent amounts of either FK or cold CsA (1:1, wt/wt) together with [^{3}H] CsA at each point in the incubation medium on the cellular uptake of [^{3}H] CsA (panel A). For panels B and C, the experimental conditions were identical to those in panel A except that cells were preincubated for 24 hours with 10 μg FK/mL (panel C). Subsequently, the cells were washed and incubated with [^{3}H] CsA as described above. Under all three conditions, the cellular uptake of [^{3}H] CsA was higher at each concentration of [^{3}H] CsA in the incubation in the presence of equivalent amounts of FK than it was in its absence. When the cells were preincubated with 10 μg CsA, the absolute uptake of [^{3}H] CsA was reduced in all cases relative to panels A and C, suggesting that some of the cellular CsA binding sites are taken up by unlabeled CsA. However, even under these conditions, inclusion of FK in the medium was able to induce a higher [^{3}H] CsA uptake (Fig 3, panels A, B, and C) compared with unstimulated cells. Conditions for these experiments were identical to those for the experiments in Fig 3, except that preincubations with 10 μg CsA and 1 μg FK/mL of medium were for 72 hours.

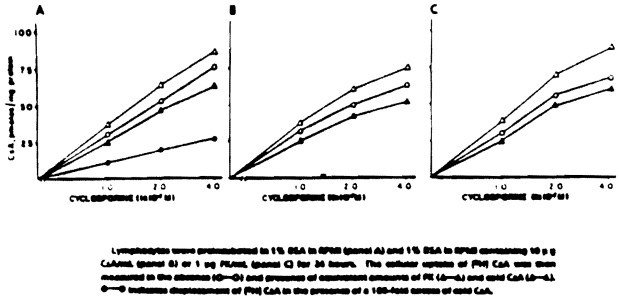

Fig 3.

Again, the uptake of [^{3}H] CsA is enhanced when the incubations contain amounts of FK equivalent to the amounts of [^{3}H] CsA at each point.

DISCUSSION

Cyclosporine and FK506 share a remarkable array of properties despite the obvious differences in their molecular structures. Although the immunosuppressive effects of FK506 are several hundredfold greater than those of cyclosporine A, the mechanism of immunosuppression of the two drugs is the same, i.e., blockage of interleukin release. This report delineates further similarities between the two drugs. Both drugs exhibit saturation kinetics of binding to human peripheral blood lymphocytes, and both apparently bind to the same intracellular protein. If it is indeed the same protein that both the drugs are bound to, the question of whether separate binding sites are involved in this process must be investigated. Our results also demonstrate that human peripheral blood lymphocytes exposed to FK506 are able to take up more cyclosporine relative to cells that are not similarly exposed. Their sensitivity to cyclosporine in terms of MLC and primed lymphocyte tests is also increased. This evidence suggests that binding of one drug, FK506, appears to modify the cellular response to another drug, cyclosporine, resulting in a greater uptake of cyclosporine by the cell.

CONCLUSIONS

The binding and kinetics of uptake of FK506 by human peripheral blood lymphocyte exhibit a saturable process, with saturation occurring at a concentration of approximately 0.5 μM of the drug.

Skatchard analysis of binding data (Fig 1) indicates two distinct classes of binding sites for FK506: (1) $K_d = 3.9 \pm 1.8 \times 10^{-9}$M, high affinity, and (2) $K_d = 5.2 \pm 0.8 \times 10^{-6}$M, low affinity.

Intracellularly, both drugs appear to bind to a common protein with an apparent molecular weight of 18,000 to 19,000 dalton.

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