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IN VIVO MECHANISMS FOR THE INHIBITION OF T LYMPHOCYTE ACTIVATION BY LONG-TERM THERAPY WITH TACROLIMUS (FK-506)

Experience in Patients with Behçet's Disease

NOBORU SUZUKI, SAKAE KANEKO, MOTOHIDE ICHINO, SHOJI MIHARA, SUESHIGE WAKISAKA, and TSUYOSHI SAKANE

Objective. **To examine the in vivo mechanisms of suppression of T lymphocyte function in patients with Behçet's disease (BD) undergoing long-term treatment with tacrolimus (FK-506).**

Methods. **Intracellular proteins were analyzed by immunoprecipitation and Western blotting. Messenger RNA expression was studied by a polymerase chain reaction–based technique.**

Results. **Interleukin-2 production was suppressed in patients treated with tacrolimus. This suppression was found to be due to inhibition of interactions between activated calcineurin (Cn) and nuclear factor of activated T cells (NF-AT), inhibition of cleavage of the autoinhibitory domain of the CnA subunit, and inhibition of heterodimer formation by CnA and CnB subunits, resulting in the absence of NF-AT in nuclei of the T cells. We found that T lymphocytes in some BD patients treated with tacrolimus had reduced amounts of FK-506 binding protein (FKBP) in their cytoplasm.**

Conclusion. **Tacrolimus reduces the Cn activity of T cells in vivo by the cumulative effects of several distinct mechanisms. It is plausible that reduced amounts of FKBP may be associated with diminished clinical efficacy in some BD patients receiving prolonged treatment with tacrolimus.**

Tacrolimus (FK-506) has assumed a major role in the treatment of allograft rejection and autoimmune and allergic diseases. Its use is based on its profound effects on immune responses (1–4). In spite of its possible widespread medical use, the mechanism of its inhibitory effects has not been completely elucidated. Several recent studies have explored this area, using transformed lymphocytes, brain extracts containing calcineurin (Cn), or a purified/recombinant enzyme (5–9). However, the mechanisms of FK-506–mediated inhibition of human T cell function in patients with various immunologic disorders who are receiving this medication remain to be elucidated.

Behçet's disease (BD) is a systemic inflammatory disease of unknown etiology (10–12), characterized mainly by recurrent oral and genital ulcerations, skin lesions, and uveitis. Viral, genetic, and environmental factors have been implicated in the pathogenesis of the disease (13,14). In patients with BD, several T cell abnormalities that may be quite relevant to the autoimmune origin of the disease have also been described (15–20). Recently, it was reported that significant lymphoproliferative responses were induced with 4 specific peptides derived from the 65-kd mycobacterial heat shock protein (HSP) and the homologous sequences of the 60-kd human HSP in patients with BD (21,22). HSP has been reported to be involved in the pathogenesis of T cell–mediated autoimmune diseases, including rheumatoid arthritis and insulin-dependent diabetes mellitus

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Noboru Suzuki, MD, PhD, Sakae Kaneko, MD, Motohide Ichino, PhD, Shoji Mihara, MD, Sueshige Wakisaka, MD, Tsuyoshi Sakane, MD, PhD: St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.

Address reprint requests to Tsuyoshi Sakane, MD, PhD, Departments of Immunology and Medicine, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216, Japan.

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(23–25). These results suggest a similarity between autoimmune diseases and BD.

Because immune abnormalities are intimately associated with the development of BD, FK-506 has been used to treat patients with BD in multicenter, open, clinical trials in Japan (26,27). As a result, it has been shown that long-term therapy with FK-506 in BD patients is effective in suppressing the activity of uveitis and some of the other systemic symptoms and in maintaining visual acuity in most patients with BD, whereas previous therapy with various immunosuppressive agents, including cyclosporin A, had failed to manage the ocular manifestations of the disease (26,27). These reports prompted us to study the mechanisms of FK-506 in patients with BD. In the present study, we addressed the in vivo mechanisms responsible for the immunosuppressive effects on T cell function in patients with BD receiving treatment with FK-506. We found that administration of FK-506 reduces the Cn activity of T cells in vivo in patients with BD by the cumulative effects of several distinct mechanisms.

PATIENTS AND METHODS

Patients and controls. Sixteen patients (10 men and 6 women) who satisfied the 1987 diagnostic criteria for Behçet's disease proposed by the Behçet's Disease Research Committee of Japan (28) were studied. They also fulfilled a criterion for the diagnosis of BD by the International Study Group for Behçet's Disease (29). Recurrent oral ulceration, recurrent genital ulceration, eye lesions, and skin lesions are common items of the 2 sets of diagnostic criteria. In addition, a positive pathergy test is included as 1 of 5 items of the International Study Group's criteria.

An open clinical trial of FK-506 in 40 BD patients with refractory uveitis from 9 institutes including St. Marianna University Hospital (Kawasaki, Japan) was conducted from December 1990 to April 1992. Clinical effectiveness was determined on the basis of any improvement or deterioration in symptoms after 12 weeks of FK-506 administration. Patients who were successfully treated with FK-506 in this trial were continued on this medication regimen until March 1995. The results of the clinical trial, which show the effectiveness of FK-506 in refractory uveitis in patients with BD, will be published in an appropriate journal soon.

For the present study, we evaluated 8 patients from St. Marianna University Hospital who were part of the clinical trial. All 8 patients had responded to the FK-506 treatment, showing completely inactive disease while receiving 0.05–0.15 mg/kg/day of FK-506 during that study. In addition, blood sampling from the same patients was repeated; the patients who took FK-506 for >3 months and then had blood drawn (while taking FK-506), were off the drug for >3 months, and then had blood drawn for the second time (off FK-506). As a patient control population, we selected 8 BD patients with completely inactive disease, who had never been treated with FK-506. They were receiving 1 mg/day of colchicine and/or nonsteroidal antiinflammatory drugs at the time of study. Patients who were taking glucocorticoids and/or cytotoxic drugs were excluded from this study.

Plasma concentrations of FK-506 in BD patients taking a dosage of 0.075 mg/kg/day of FK-506 orally were ~1–10 ng/ml. Their mean ± SD age was 36 ± 11 years (range 22–54). Twelve healthy volunteer blood donors (7 men and 5 women) served as normal control subjects; their mean age was 35 ± 7 years (range 24–46). The Human Studies Committee's approval and the informed consent of each patient were obtained before we conducted the present study.

Reagents. Phytohemagglutinin (PHA) was purchased from Murex Diagnostics (Dartford, UK). The antibodies used in this study included polyclonal anti-CnA+CnB subunits, polyclonal anti-CnB subunit, and monoclonal anti-CnB subunit (all from Upstate Biotechnology, Lake Placid, NY) and monoclonal anti–FKBP-12 antibody (kindly provided by Fujisawa Pharmaceuticals, Osaka, Japan) (30). Polyclonal anti-cyclophilin A antibody was purchased from Affinity Bioreagents (Neshanic Station, NJ). We also developed anti-human NF-AT cytoplasmic antibody by immunizing rabbits with the peptide IFLTVSREHERVGCFF (31), and we used this antibody for the immunoprecipitation study.

Cell separation and cell culture. Peripheral blood mononuclear cells obtained by Ficoll-Hypaque gradient centrifugation were depleted of monocytes by adherence to plastic dishes (32). Monocyte-depleted cells were separated into T cells and non–T cells by a sheep red blood cell rosetting technique (32). Non–T cells were cultured on plastic dishes and nonadherent cells were removed. Adherent cells were recovered as monocytes. T cells plus 5% irradiated autologous monocytes were resuspended at a cell density of 1×10^6/ml in medium consisting of RPMI 1640 containing penicillin (100 μg/ml) and streptomycin (100 units/ml) (Life Technologies, Gaithersburg, MD) and 10% fetal calf serum (Life Technologies), and were cultured with 1 μg/ml of PHA at 37°C in 5% CO_2/95% air for various times. In some experiments, cells were preincubated for 30 minutes in vitro with or without 0.1–100 ng/ml of FK-506 before stimulation.

In parallel studies, we found that there were no alterations in the lymphocyte subpopulations at the dosage of FK-506 used for this study protocol. The following values for subpopulations were obtained (by flow cytometry) just before and after >3 months of FK-506 treatment in 40 patients: CD2-positive cells 86.1 ± 5.7% (mean ± SD) and 84.6 ± 5.8%; CD3-positive cells 74.1 ± 9.2% and 72.8 ± 8.0%; CD4-positive cells 44.6 ± 10.9% and 41.5 ± 10.1%; CD8-positive cells 29.8 ± 7.7% and 31.0 ± 8.2%; and CD20-positive cells 4.5 ± 2.9% and 5.0 ± 3.0%. There were no statistically significant differences in these values.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. Levels of interleukin-2 (IL-2) messenger RNA (mRNA) and FK-506 binding protein (FKBP) mRNA expression by the T cells were estimated by a PCR-based technique (33,34). Briefly, total RNA was extracted from freshly isolated T cells or T cells cultured with PHA (1 μg/ml) for 6 hours, and complementary DNA (cDNA) was synthesized. β-actin primers were used to compare and monitor efficient cDNA synthesis between different samples. The primers were as follows: β-actin (548 basepairs) 5′ primer GTGGGGCGCCCCAGGCAC, 3′ primer CTCCTTAATGTCACGCACGAT; IL-2 (461 bp) 5′ primer ATGTACAGGATGCAACTCCTG, 3′

primer CAAGTCAGTGTTGAGATGATGC; and FKBP (327 bp) 5′ primer ATGGGAGTGCAGGTGGAAAC, 3′ primer TCATTCCAGTTTTAGAAGCTCC. For all reactions, temperature cycling was at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, for 30 cycles, followed by a cycle of 72°C for 10 minutes.

Enzyme-linked immunosorbent assay (ELISA) for IL-2. IL-2 levels in culture supernatants were measured by an IL-2 ELISA (Otsuka Assay Co., Tokushima, Japan). The lower limit of detection for this assay was 50 pg/ml.

Measurement of Cn phosphatase activity. Cn was immunoprecipitated from T cells by anti-CnB subunit antibody using Ca^{++} and Mg^{++}–free phosphate buffered saline supplemented with 0.2% Nonidet P40 (a nonionic detergent; Sigma, St. Louis, MO). We included 50 μg/ml of phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml of soybean trypsin inhibitor, 10 μg/ml of leupeptin, and 10 μg/ml of aprotinin as protease inhibitors. Thereafter, Cn phosphatase activity was assayed spectrophotometrically by following the hydrolysis of *p*-nitrophenyl phosphate (Sigma). Briefly, 60 m*M* *p*-nitrophenyl phosphate in the buffer (50 m*M* Tris, pH 7.8, 0.1 m*M* EGTA, 0.1% 2-mercaptoethanol [2-ME]) was reacted with immunoprecipitated Cn, followed by measuring the change in absorbance at 405 nm (35). (The results presented in Figure 3 represent optical density at 15 minutes.) This assay system does not cross-react with phosphatases 1 and 2A, since introduction of 500 n*M* okadaic acid, an inhibitor of protein phosphatases 1 and 2A, did not affect the results (data not shown).

In addition, magnesium was excluded from the assay buffer to minimize the activity of phosphatase 2C. Liu et al (36) reported that FK-506–FKBP-12 complexes formed in vitro in the presence of Ca^{++} and Mg^{++} induce a slight increase in the phosphatase activity of Cn–Ca^{++}–calmodulin toward *p*-nitrophenyl phosphate (36). However, in our preliminary experiments, we found that Cn purified by this experimental condition, where Ca^{++} and Mg^{++} are excluded, gives a relevant activity in the spectrometric assay.

Western blotting. T cells were lysed with buffer containing 50 m*M* Tris, pH 8.0, 1% Nonidet P40, 150 m*M* NaCl, and the protease inhibitors. Equivalent amounts of proteins from the patients were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked with 3.5% bovine serum albumin. Western blots were performed using the antibodies described above. Blots were probed with appropriate biotin-conjugated secondary antibody, followed by streptavidin–alkaline phosphatase and detection by nitroblue tetrazolium–BCIP–toluidine salt (Sigma) or chemiluminescence.

Analysis of immunoprecipitates. T cells were suspended in lysis buffer, consisting of 50 m*M* Tris, pH 7.5, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 1% Nonidet P40, and the protease inhibitors. The lysates were precleared 3 times with nonimmune normal mouse or rabbit serum and packed protein A–Sepharose beads for 2 hours with rotation, followed by incubation with the appropriate antibody for 2 hours at 4°C. Protein A–Sepharose beads were then added. Proteins were eluted from protein A–Sepharose by boiling for 5 minutes in buffer containing 1% SDS in the presence or absence of 5% 2-ME and analyzed on SDS-polyacrylamide gels (37). (The gels shown in Figures 4, 5, and 8 were run under reducing conditions.)

Nuclear extracts. Nuclear extracts were prepared from T cells by a modification of the method of Dignam et al (38). Briefly, cells were homogenized in 2 cell pellet volumes of 10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 10% glycerol, and the protease inhibitors (38,39). The resultant nuclear pellet was homogenized in 2 cell pellet volumes of 20 m*M* HEPES, pH 7.9, 0.42*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 25% glycerol, and the protease inhibitors. After 30 minutes of incubation at 4°C, the samples were centrifuged for 20 minutes, and the supernatants were dialyzed against buffer consisting of 20 m*M* HEPES, 20% glycerol, 0.2 m*M* EDTA, 0.5 m*M* PMSF, and 0.5 m*M* DTT. The protein concentration in the nuclear extracts was determined by the Bradford assay (40).

DNA protein-binding assay. Gel-shift assay was performed using a digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions (41,42). Briefly, DIG-labeled DNA fragments were incubated at room temperature for 15 minutes with 5–10 μg of nuclear proteins. Protein–DNA complexes were separated from free probe on a polyacrylamide gel. Thereafter, the gels were electrically transferred to nylon membrane, and the complexes were detected by chemiluminescence. We verified that a 20-fold excess of specific cold oligonucleotide would compete the binding of the protein to the DIG-labeled probe (see Figure 6), whereas a similar excess from another site would not compete (data not shown).

DNA probes. The probe was derived from sequence present in the IL-2 promoter region (43). The NF-AT binding site from −285 to −254, GGAGGAAAAACTGTTTCATACAGAAGGCGT (44) was labeled with DIG by using a 3′-end labeling kit (Boehringer).

RESULTS

IL-2 synthesis of T cells from patients with BD receiving FK-506 therapy. We have previously shown that T cells from patients with Behçet's disease who are taking neither cyclosporin A nor FK-506 produce normal amounts of IL-2 upon mitogen stimulation; however, IL-2 receptor expression by the T cells is defective (7). We determined whether T cells from patients with BD undergoing treatment with FK-506 produce IL-2 in response to PHA. We found that IL-2 production by these cells was completely suppressed (Figure 1). However, T cells from patients with BD who were no longer receiving FK-506 (FK-506 taken for >3 months; other treatment for >3 months; then blood sampling for this study) secreted almost normal amounts of IL-2 upon stimulation. This suppression of IL-2 protein production may be due to the impaired monocyte function. However, we found that in vitro pretreatment of autologous monocytes, but not T lymphocytes, with FK-506 did not inhibit T cell proliferative responses in normal individ-

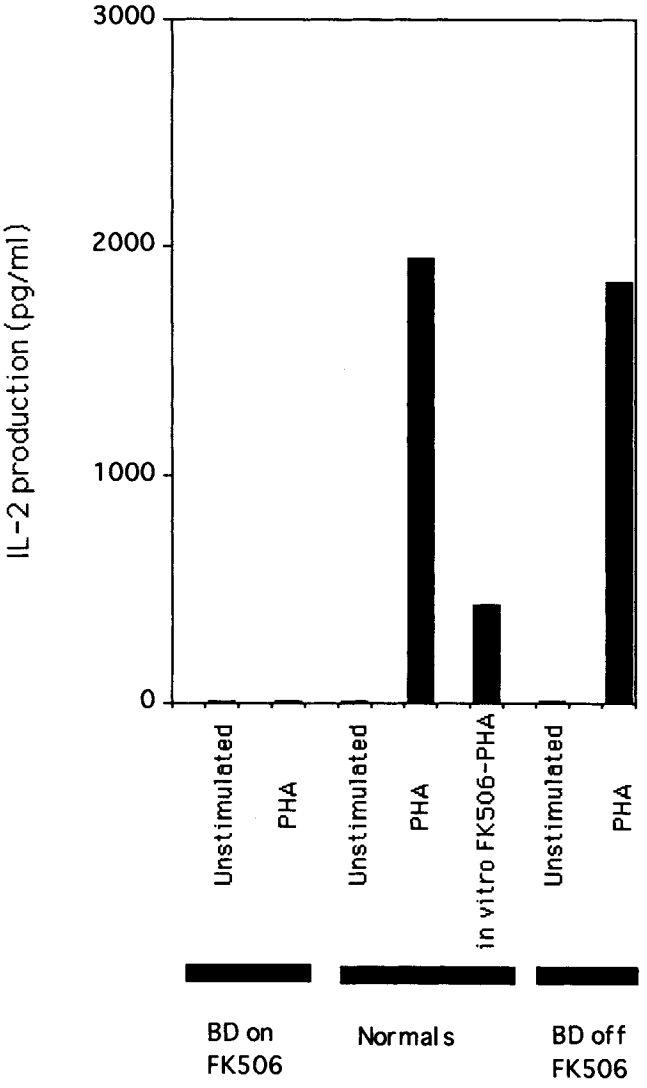

Figure 1. Interleukin-2 (IL-2) production by T cells from FK-506–treated patients with Behçet's disease (BD). T cells were recovered from the blood of BD patients who had been treated with FK-506 for >3 months. T cells + 5% autologous monocytes were stimulated for 24 hours with 1 μg/ml of phytohemagglutinin (PHA). Normal T cells were pretreated in vitro for 30 minutes with 10 ng/ml of FK-506 and then stimulated with PHA. The SEM did not exceed 15% of the means for each group of subjects (n = 5 per group).

uals (T cells + 5% monocytes in medium 300 counts per minute, in 1 μg/ml PHA 26,700 cpm, in 1 μg/ml PHA plus 10 ng/ml FK-506 6,200 cpm; T cells + 5% monocytes pretreated with 10 ng/ml of FK-506 in medium 300 cpm, in 1 μg/ml PHA 22,900 cpm).

We also found that T cell responses in patients with BD who had been taking FK-506 were defective even when anti-CD3 antibody fixed to the plate was used as a stimulus (normal unstimulated T cells 600 cpm; FK-506–treated BD patients unstimulated T cells 500 cpm; normal stimulated T cells 15,200 cpm; FK-506–treated BD patients stimulated T cells 2,900 cpm). In addition, monocytes from FK-506–treated BD patients exhibited antigen-presenting cell function to allogeneic normal T cells when PHA was used (normal unstimulated T cells 700 cpm; normal PHA-stimulated T cells with 5% autologous monocytes 64,700 cpm; normal PHA-stimulated T cells with monocytes from an FK-506–treated patient 53,900 cpm). Thus, it is highly unlikely that our observation is due to a defect in monocytes alone. Rather, these results support our notion that the T cells are responsible for immunosuppression in BD patients taking FK-506.

This suppression of IL-2 protein synthesis occurred at the mRNA level, since we did not detect any IL-2 mRNA by RT-PCR in T cells from FK-506–treated BD patients (Figure 2). It should be noted that in vitro

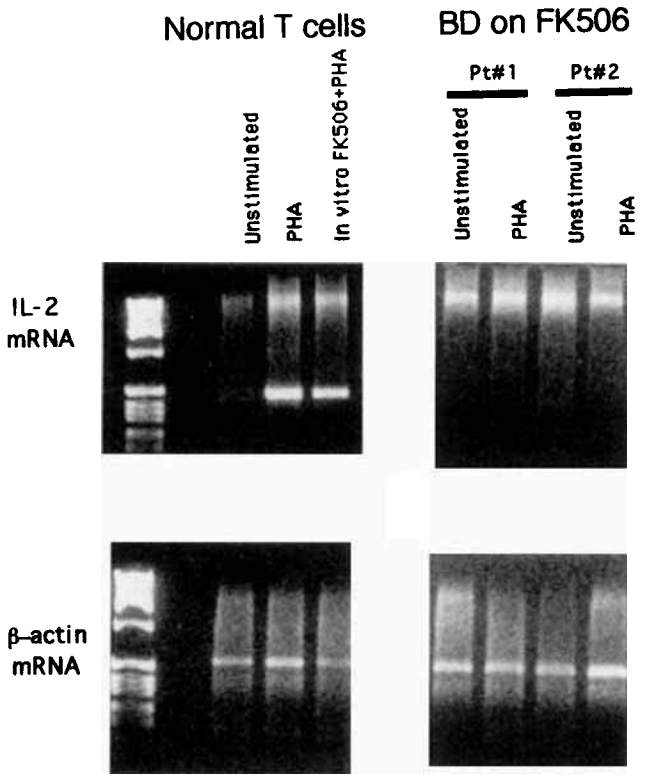

Figure 2. IL-2 messenger RNA (mRNA) expression by T cells from FK-506–treated patients with BD. T cells + 5% autologous monocytes from BD patients who had been treated with FK-506 for >3 months and from normal subjects were stimulated for 6 hours with 1 μg/ml of PHA. The normal T cells + 5% autologous monocytes were also pretreated in vitro for 30 minutes with 10 ng/ml of FK-506 and then stimulated with PHA. IL-2 mRNA expression was estimated after reverse transcription–polymerase chain reaction. A1-kb DNA ladder (Life Technologies) is shown in the far left lanes. See Figure 1 for other definitions.

treatment of normal T cells with FK-506 reduced, but did not totally suppress, IL-2 mRNA expression (Figure 2). We also found that T cells from FK-506–treated BD patients did not produce IL-2; nonetheless, they responded to exogenous IL-2 added to the in vitro cell culture (data not shown).

Cn phosphatase activity of T cells in FK-506–treated BD patients. It was important to determine whether the Cn phosphatase activity of T cells was really suppressed in FK-506–treated BD patients. The Cn phosphatase activity of the T cells was assayed spectrophotometrically by following hydrolysis of *p*-nitrophenyl phosphate, using Cn that was immunoprecipitated by anti-CnB antibody from the T cells of FK-506–treated BD patients. The Cn phosphatase activity of the PHA-stimulated T cells from FK-506–treated BD patients was completely suppressed (Figure 3). In contrast, immunoprecipitates of T cells from patients with BD who were not treated with FK-506 as well as from normal subjects showed a significant phosphatase activity upon PHA stimulation (Figure 3). As expected, Cn phosphatase activity of PHA-stimulated normal T cells treated with FK-506 was profoundly suppressed (Figure 3).

Molecular characterization of deficient Cn activity in FK-506–treated BD patients. To clarify what mechanisms are responsible for the defective Cn phosphatase activity in FK-506–treated BD patients, we conducted immunoprecipitation analyses of Cn and NF-AT from the T cells. It has been reported that FK-506 interacts with FKBP-12 of T cells and forms an FK-506–FKBP complex, which can then bind to Cn, inhibit its enzymatic activity, and thereby prevent early gene expression (21–25).

T cells from BD patients or from normal subjects were stimulated with PHA for 1–3 hours, and the T cell lysates were incubated with anti-CnB subunit antibody. As shown in Figure 4A, similar amounts of CnB were immunoprecipitated from unstimulated normal T cells, PHA-stimulated T cells, and in vitro FK-506 + PHA–treated T cells. In contrast, CnA that was coimmunoprecipitated by anti-CnB antibody was scarcely detected in unstimulated T cells (large arrow, Figure 4A). Small CnA that had been cleaved from its autoinhibitory domain was prominent in PHA-stimulated T cells. In vitro FK-506 treatment partially prevented cleavage of the autoinhibitory domain, resulting in the appearance of 2 different size CnA subunits. Thus, PHA stimulation induced coimmunoprecipitation of CnA subunit with CnB subunit by using anti-CnB antibody in normal T cells, which suggests that heterodimer formation of CnA and CnB can be enhanced by mitogenic stimulation of normal T cells, and FK-506 inhibited cleavage of the autoinhibitory domain of CnA. However, PHA stimulation of T cells from FK-506–treated BD patients did not enhance the coimmunoprecipitation of the CnA subunit with the CnB subunit by using anti-CnB antibody. Thus, it is evident that heterodimer formation of CnA and CnB upon cell activation is inhibited in T cells from FK-506–treated BD patients.

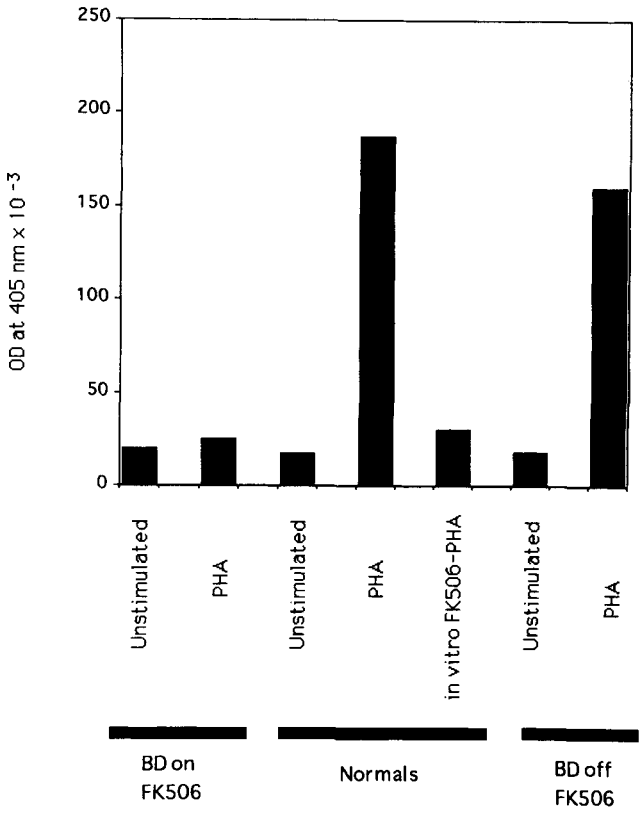

Figure 3. Calcineurin (Cn) phosphatase activity in T cells from FK-506–treated patients with BD. T cells were recovered from the blood of BD patients who had been treated with FK-506 for >3 months. T cells + 5% autologous monocytes were stimulated for 3 hours with 1 μg/ml of PHA. Normal T cells were pretreated in vitro for 30 minutes with 10 ng/ml of FK-506 and then stimulated with PHA. Cn was immunoprecipitated and the Cn phosphatase activity of the T cells was measured. The SEM did not exceed 15% of the means for each group of subjects (n = 5 per group). See Figure 1 for other definitions.

It has been shown that complexes of CnA and CnB have been formed in nerve cells and kidney cells (36,45,46) without cell activation. In the present study, we have also found that some, but not all, of the CnA and CnB subunits are present separately in resting T lymphocytes and that they form heterodimers upon activation (Figure 4A, left panel). The reason for this difference is not clear. One possibility is that complex formation by CnA and CnB upon activation reduces background Cn activity in the resting state; thus, T cells transduce clear signals to the nucleus when T cells are activated. In addition, cleavage of the autoinhibitory

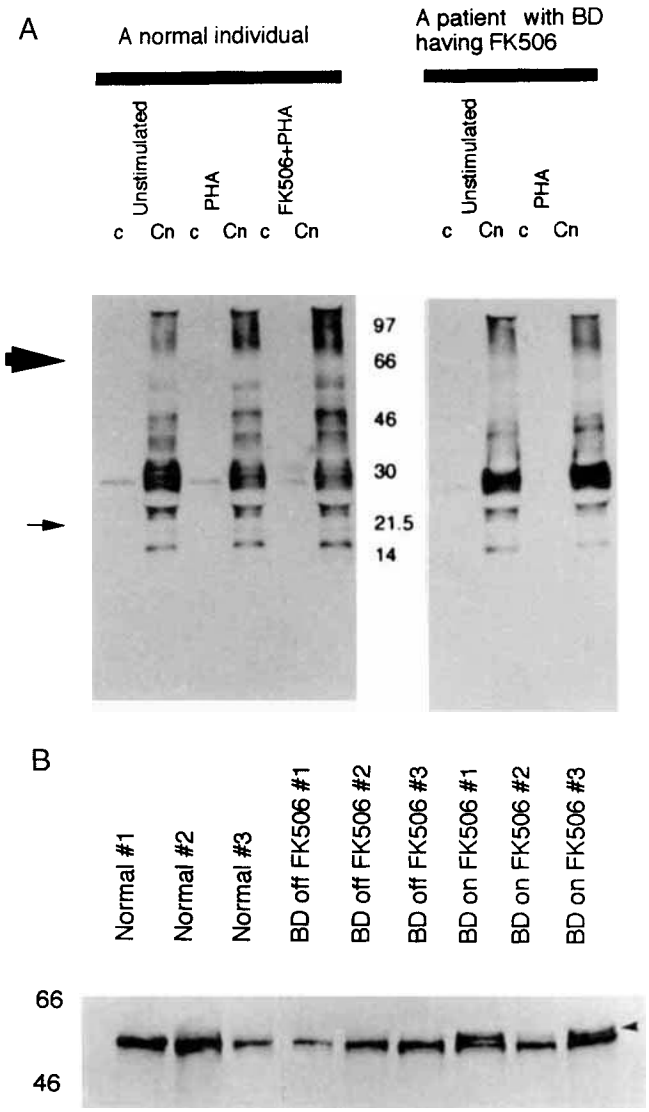

Figure 4. Immunochemical study of calcineurin (Cn) in T lymphocytes. **A,** Immunoprecipitation of Cn. T cells from BD patients who had been treated with FK-506 for >3 months were stimulated for 3 hours with 1 μg/ml of PHA. Normal T cells were pretreated in vitro for 30 minutes with 10 ng/ml of FK-506 and then stimulated with PHA. T cells were lysed in buffer containing Ca^{++} and Mg^{++}. Immunoprecipitation was carried out using anti-CnB antibody and purified mouse IgG as a control, and cells were then Western blotted onto the membrane. The blot was probed with anti-CnA+CnB antibody, followed by biotin anti-rabbit IgG antibody and streptavidin–alkaline phosphatase. CnB **(small arrow)** and CnA **(large arrow)** markers are shown to the left. Similar results were obtained in 2 different sets of experiments. **B,** Western blotting analysis of CnA in T cells from FK-506–treated BD patients. Patients' T cells were stimulated for 3 hours with 1 μg/ml of PHA, and the T cells were lysed and Western blotted onto the membrane. The blot was probed with anti-CnA+CnB antibody. Small CnA, which lacks the autoinhibitory domain, is seen in the normal subjects, the BD patients off FK-506 (>3 months after treatment termination), and patients with BD taking FK-506 (>3 months). In contrast, large CnA, which has the autoinhibitory domain, was detected only in the patients receiving FK-506. Results are representative of 3 different experiments. See Figure 1 for other definitions.

domain of CnA on PHA stimulation is at least partly inhibited in FK-506–treated BD patients (in addition to the small CnA, large CnA was evident in BD patients 1 and 3, and cells from BD patient 2 contained small amounts of large CnA [see Figure 4B]), as was shown in PHA-stimulated normal T cells pretreated with FK-506 in vitro (Figure 4A).

In contrast, PHA-stimulated T cells from BD patients who had never been treated with FK-506 and from normal subjects had CnA that lacked the autoinhibitory domain (small CnA in Figure 4B).

We next conducted immunoprecipitation analysis using anti-NF-ATc antibody. As shown in Figure 5, PHA-activated normal T cells contained ~100-kd NF-ATc (31) in the cytoplasm. Resting normal T cells did not contain detectable amounts of NF-ATc in the present experiments. The CnA subunit was coimmunoprecipitated with NF-ATc when the cells were PHA activated. In contrast, T cells from FK-506–treated BD patients did not contain a sufficient amount of the ~100-kd NF-ATc. In addition, coimmunoprecipitation of CnA with NF-ATc was not detected. Thus, amounts of NF-ATc present in the cytoplasm of T cells from BD patients who received FK-506 for >3 months were substantially reduced compared with the amounts present in normal T cells. Furthermore FK-506, directly or indirectly, prevents complex formation by Cn and NF-ATc, which is required for dephosphorylation of NF-ATc.

Thus, it is suggested that suppression of IL-2 production in FK-506–treated BD patients is due to the cumulative effects of the following events: 1) inhibition of essential interactions between activated Cn and NF-AT that is reduced in FK-506–treated BD patients; 2) inhibition of heterodimer formation consisting of CnA and CnB subunits; and 3) FK-506 inhibition of cleavage of the autoinhibitory domain of CnA upon activation. We also found that FK-506 suppresses CnB subunit synthesis by normal T cells in vitro (data not shown). However, we do not understand the in vivo significance of this finding, since T cells from FK-506–treated BD patients have considerable amounts of CnB in their cytoplasm (Figure 4A).

Absence of NF-AT in the nuclei of activated T cells from FK-506–treated BD patients. To confirm that FK-506 treatment of patients with BD prevents T cell activation in vivo, we analyzed NF-AT in the T cell nuclei by gel shift assay (47). It has been reported that unstimulated circulating human T cells contain no detectable NF-AT, and that NF-AT appears within 6 hours upon mitogenic stimulation (48). As shown in Figure 6, NF-AT was not detected in the T cells from FK-506–

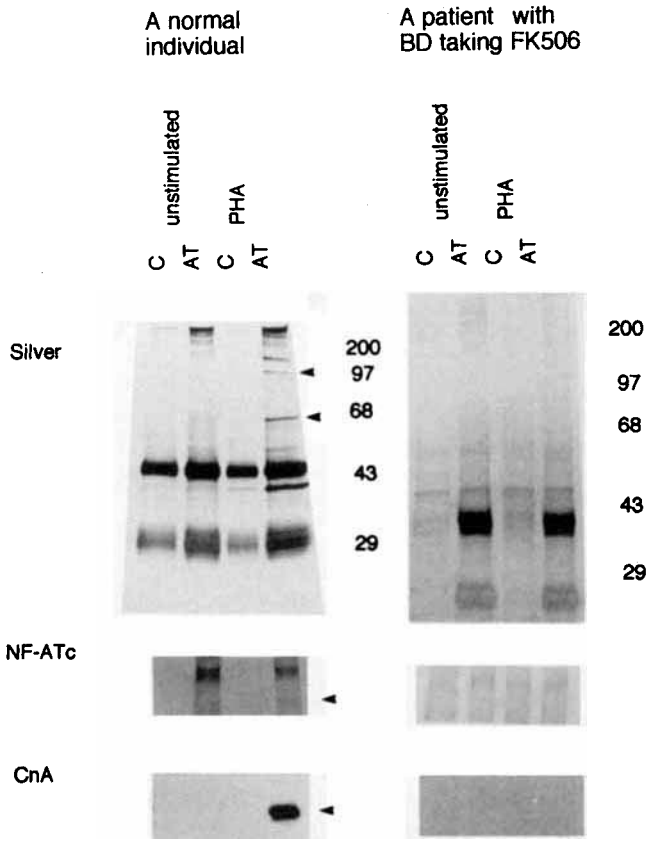

Figure 5. Immunoprecipitation study of nuclear factor of activated T cells (NF-AT) in T lymphocytes. Normal T cells and T cells from BD patients treated with FK-506 for >3 months were stimulated for 3 hours with 1 μg/ml of PHA. T cells were lysed with buffer containing Ca^{++} and Mg^{++}. Immunoprecipitation was carried out using rabbit polyclonal anti–NF-ATc antibody and purified rabbit IgG as a control, and cells were Western blotted onto the membrane. The blot was probed with anti–NF-AT cytoplasmic (anti–NF-ATc) antibody or anti-CnA antibody. NF-ATc and CnA **(arrowheads)** are shown in the middle. Approximately 100-kd NF-ATc was detected in PHA-stimulated normal T cells, but not in BD patients' T cells. In addition, CnA was coimmunoprecipitated in PHA-stimulated normal, but not BD, T cells by anti–NF-ATc antibody. Results are representative of 3 different experiments. Immunoprecipitated with control (C) IgG and anti–TNF-ATc antibody (AT). See Figure 1 for other definitions.

treated BD patients, even after stimulation with PHA. It is therefore suggested that inhibition of Cn activity by FK-506 results in the absence of the activated form of NF-AT in the T cell nuclei. Thus, IL-2 production was reduced in FK-506–treated BD patients.

Variations in FKBP-12 levels in T cells from patients with BD. We next examined whether there is any variation or change in FKBP levels in patients with BD. It has been reported that enhanced expression of FKBP-12 results in increased sensitivity to FK-506 in some cell lines (49–51), which suggests that decreased sensitivity to (effectiveness of) FK-506 may be related to altered expression of FKBP. We analyzed FKBP mRNA expression by fresh, unstimulated T cells by RT-PCR (Figure 7). To our surprise, FKBP mRNA expression was lacking in some BD patients during their treatment. Patient 3, who was evaluated 4 times, had relatively stable FKBP mRNA expression, regardless of whether he was taking FK-506 (lanes 7–10, Figure 7). Patients 4 and 2 were evaluated 3 and 2 times, respectively. Patient 4 had abundant FKBP mRNA expression before starting FK-506 treatment (lane 4). However, the mRNA expression was not detected during long-term FK-506 therapy (10 months; lane 5). Similarly, patient 2, who lacked FKBP mRNA expression during long-term FK-506 therapy (2 years; lane 11), showed a return to normal levels of FKBP mRNA expression 10 months after termination of FK-506 (lane 12). In contrast, normal individuals had relatively stable FKBP mRNA expression.

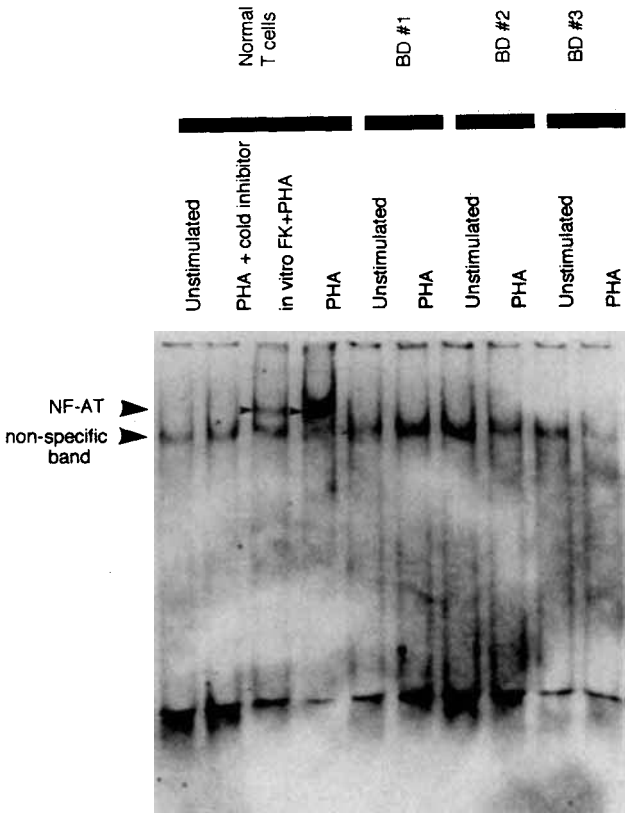

Figure 6. Nuclear factor of activated T cells (NF-AT) activity in T cells from FK-506–treated BD patients. Nuclear proteins from resting or PHA-stimulated T cells were analyzed by gel shift assay. A digoxigenin-labeled NF-AT probe was used. Results are representative of 3 different experiments. NF-AT was also detected in PHA-stimulated T cells from BD patients who were not being treated with FK-506 (data not shown).

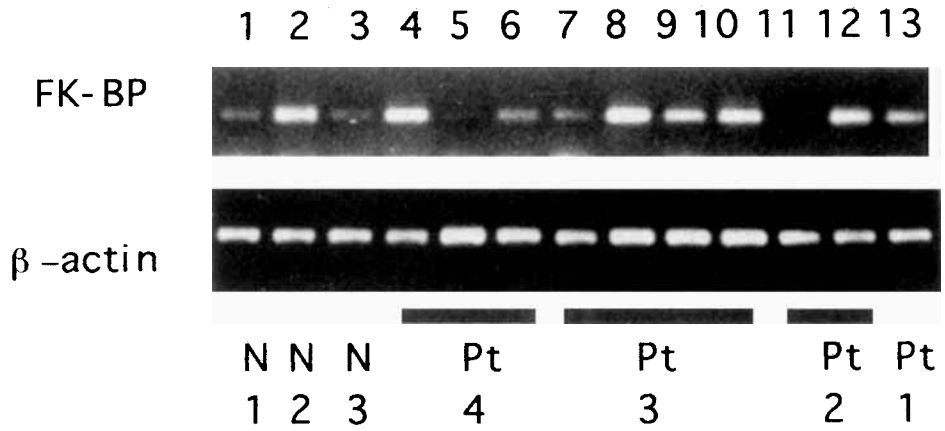

Figure 7. Expression of FK-506 binding protein (FKBP-12) messenger RNA (mRNA) in T lymphocytes from BD patients. Freshly isolated T cells were purified and subjected to reverse transcription–polymerase chain reaction. Some of the BD patients did not express FKBP-12 mRNA in their T cells. As a control, β-actin was simultaneously amplified. Cells from patient (Pt) 4 were obtained February 1994 (lane 4), December 1994 (lane 5), and December 1995 (lane 6); cells from patient 3 were obtained March 1994 (lane 7), April 1995 (lane 8), May 1995 (lane 9), and August 1995 (lane 10); cells from patient 2 were obtained April 1994 (lane 11) and August 1995 (lane 12). Cells from 3 normal (N) subjects were run in lanes 1–3. See Figure 1 for other definitions.

FKBP protein expression by T lymphocytes was also studied by Western blotting in patients for whom sufficient T cells were available. We found that T lymphocytes from some BD patients who had not been treated with FK-506 completely lacked FKBP-12 (patient 5, Figure 8B), and T cells from some BD patients who received prolonged therapy with FK-506 (>12 months) contained reduced amounts of FKBP-12 in their cytoplasm (patients 2 and 4, Figure 8B). In contrast, comparable amounts of cyclophilin A (cyclosporin binding protein) were present in the same T cell preparations (Figure 8B). Thus, it is possible that reduced amounts of FKBP in T lymphocytes from patients with BD taking FK-506 may be associated with diminished efficacy of FK-506 after prolonged therapy, as observed in our patient population. According to the clinical trial, FK-506 was not effective for ~25% of the patients. These patients might naturally lack FKBP-12 in their T cells, as was observed in patient 5 (Figure 8).

DISCUSSION

Behçet's disease is a systemic inflammatory disorder associated with high morbidity rates for vision. It has been shown that FK-506 therapy is effective for managing the ocular manifestations of BD and for maintaining the visual acuity in most of these patients, whereas previous therapy, including cyclosporin A, had failed to control such symptoms (26,27).

In the past few years, much has been learned about the mechanism by which FK-506 inhibits T cell activation (45,46). Following the binding of FK-506 to FKBP, the complex then binds to the enzyme Cn, a Ca^{++}/calmodulin-dependent serine/threonine phosphatase, thereby inhibiting its phosphatase activity. One of the targets of Cn is NF-AT, an antigen-induced transcription factor involved in the process of T cell activation (23,25,31,45,46,52,53). Since dephosphorylation of NF-AT by Cn appears to be necessary for the translocation of NF-AT from cytoplasm to nucleus, FK-506 is thus able to prevent T cell activation by inhibiting NF-AT dephosphorylation (23,25,31,45,46,52,53). However, these studies have used transformed/tumor lymphocytes or purified Cn as an enzyme. The mechanisms of FK-506–mediated inhibition of human primary T cell function in patients with various immunologic disorders remain largely unknown.

In the present study, we investigated the requirements that govern the immunosuppression mediated by the interaction of Cn with drug–immunophilin complexes in FK-506–treated BD patients. In another study, Cn phosphatase activity in fresh, unstimulated T cells from peripheral blood obtained from bone marrow

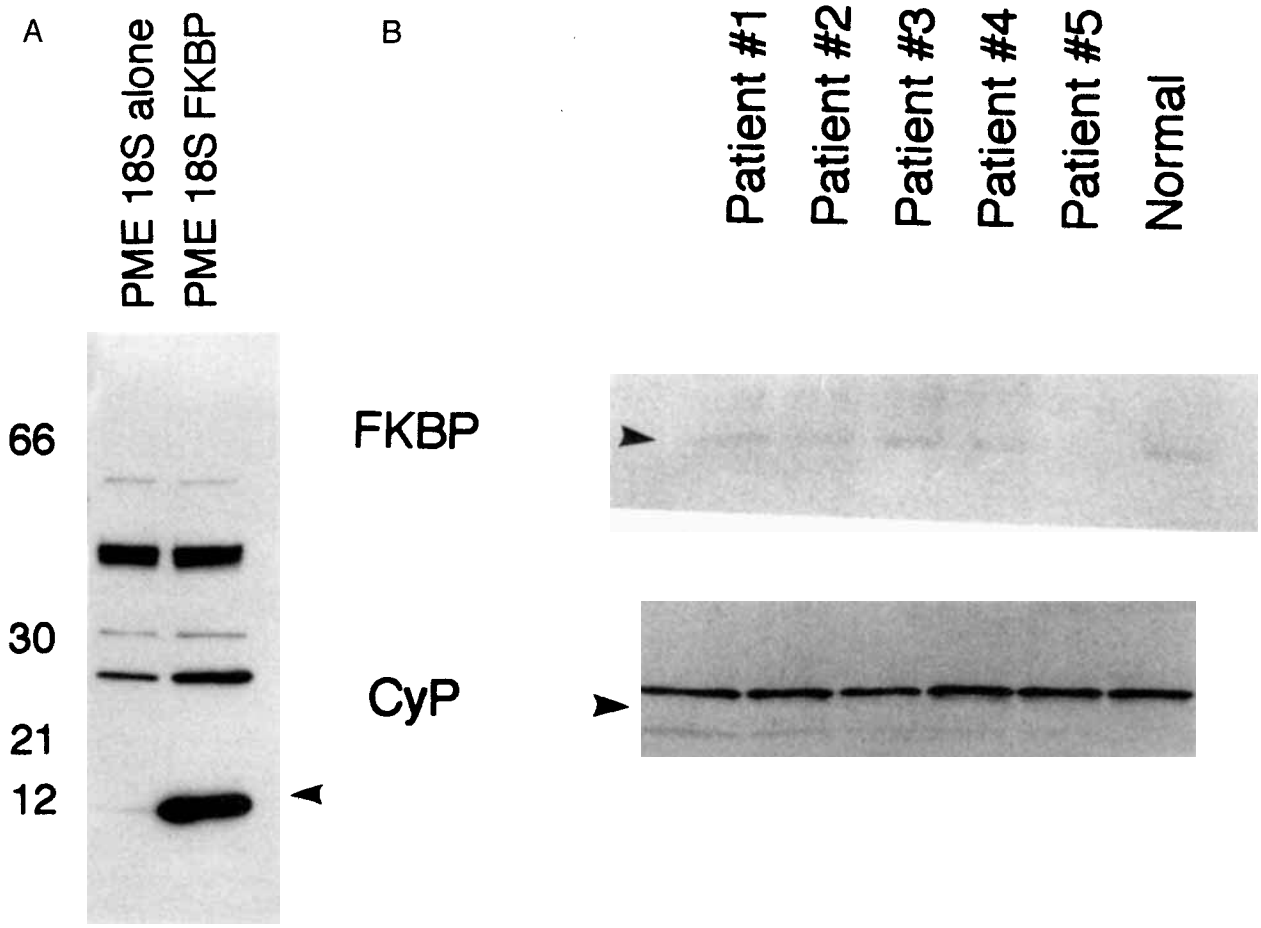

Figure 8. Expression of FK-506 binding protein (FKBP-12) in T lymphocytes from BD patients. **A,** Jurkat cells transfected with pME18S vector (kindly provided by Dr. Maruyama, University of Tokyo, Tokyo, Japan) alone or with FKBP complementary DNA were analyzed by Western blotting using anti-FKBP antibody (30). **Arrowhead** shows FKBP-12. **B,** Freshly isolated T cells were lysed and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by Western blotting and detection by anti–FKBP-12 monoclonal antibody. Some BD patients had no, or reduced amounts of, FKBP-12 in their T cells. As a control, cyclophilin A (CyP) was used. **Upper arrow** shows 12-kd FKBP; **lower arrow** shows 18-kd cyclophilin A. Results are representative of 3 different experiments. See Figure 1 for other definitions.

transplant patients receiving cyclosporin A was found to be inhibited (54). We used PHA to stimulate BD patients' T cells, since significant Cn phosphatase activity was induced in our assay method only when T cells were stimulated by mitogen, unstimulated T cells exhibiting negligible Cn phosphatase activity. The differences in T cell Cn phosphatase activity between Pai's study and ours may be due to the assay methods employed. Alternatively, the differences may reflect the activation status of T cells in vivo; T cells in bone marrow transplant patients are continuously stimulated by alloantigen; BD patients' T cells are usually not activated in vivo, as measured by spontaneous proliferation and spontaneous T cell–derived lymphokine production (16) (data not shown). T lymphocytes from FK-506–treated BD patients do not exhibit significant Cn phosphatase activity even after PHA stimulation. Thus, suppression of Cn phosphatase activity, an important step in immunosuppression by FK-506, is evident in FK-506–treated BD patients.

Cn, the site of action of the immunosuppressive drugs cyclosporin A and FK-506 is comprised of two subunits: a 59-kd catalytic subunit (CnA), which contains a calmodulin-binding domain and an autoinhibitory region, and a 19-kd intrinsic calcium-binding regulatory subunit (CnB) (55). Recent reports indicate that the presence of the B subunit is required for FK-506–FKBP to inhibit Cn (45,46,52,55,56). In fact, it has been shown that the dependence of cross-linking on the presence of

both CnA and CnB subunits (binding does not occur with recombinant B alone) as well as Ca^{++}–calmodulin gives assurance that a coordinate complex must be formed to permit cross-linkage of Cn and FK-506/FKBP (45,46,52,55,56).

Our present study shows that multiple steps are involved in the suppression of Cn phosphatase activity by FK-506 in vivo: 1) inhibition of essential interactions between activated Cn and NF-AT that is reduced in FK-506–treated BD patients; 2) inhibition of cleavage of the autoinhibitory domain of the CnA subunit; and 3) inhibition of heterodimer formation consisting of the CnA and CnB subunits. These mechanisms are not mutually exclusive, and at least some of the mechanisms may reduce Cn activity, resulting in the absence of activated form of NF-AT in the nuclei. It has been shown that FK-506, FKBP, CnA+CnB, and calmodulin form a complex that prevents Cn activity (36,45, 46,52,55,56). Nonetheless, we did not find FK-506–FKBP–CnA+CnB–calmodulin complexes in the cytoplasm of circulating T cells in FK-506–treated BD patients (data not shown). It is possible that the serum concentration of FK-506 (~1–10 ng/ml) in FK-506–treated BD patients is too low to detect such complexes. In contrast, in vitro biochemical studies used much higher concentrations of FK-506 (36,56). It has been shown that the calcium-dependent complexes of truncated CnA and CnB show significant phosphatase activity toward the substrate compared with truncated CnA alone (45,46,55). Thus, inhibition of complex formation between CnA and CnB by FK-506–FKBP complexes should result in decreased Cn phosphatase activity.

It has been shown that ~75% of patients with BD can be successfully treated with FK-506; however, the remaining 25% are refractory to this therapy (26,27). We have observed that FK-506 is useful for the treatment of patients with BD, especially at initiation of this treatment; however, its effectiveness gradually decreases with prolonged therapy (50–100 weeks) (unpublished observations). Our finding suggests that reduced amounts of FKBP in T lymphocytes from FK-506–treated BD patients may be associated with the diminished efficacy of the drug after a long period. Moreover, in some BD patients who lack FKBP in their T lymphocytes, FK-506 treatment would not be effective. Although our patient group is too small to be definitive, it clearly revealed that levels of FKBP-12 in T lymphocytes vary considerably among patients with BD, and the clinical efficacy of FK-506 varies accordingly. Such variations in FKBP levels were not noted in normal T cells. Further studies are needed before firm conclusions can be made.

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