FK-506, A NOVEL IMMUNOSUPPRESSANT ISOLATED FROM A *STREPTOMYCES*

I. FERMENTATION, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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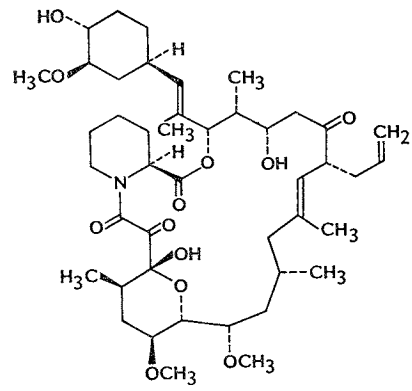
FK-506, a novel immunosuppressant, has been isolated from the fermentation broth of *Streptomyces tsukubaensis* No. 9993 as colorless prism and the molecular formula was determined as $C_{44}H_{69}NO_{12}\cdot H_2O$. The compound suppressed immune responses *in vitro* and *in vivo* with mice. This immunosuppressive effect was more potent than that of ciclosporin.

Ciclosporin (CS), a fungal metabolite, is an effective immunosuppressant with low myelotoxicity[1] and has been used successfully as the primary drug to suppress the rejection of transplants.[2~4]

It is now well established that CS inhibits the production of T cell-derived soluble mediators such as interleukin 2 (IL-2), interleukin 3 (IL-3) and gamma-interferon (IFN-γ) induced by antigens and lectins.[5~8] The immunosuppressive agents which would attack specific target sites of cells are expected to provide a useful prototype of drugs for immunotherapy. Accordingly, we have tested a wide range of fermented broths for specific inhibitory effects on IL-2 production.

Fig. 1. Structure of FK-506.



As a result, a strain of *Streptomyces tsukubaensis* No. 9993 was found to produce the potent immunosuppressive agent, designated by the code number of FK-506. Taxonomic study on this strain will be presented in a separate paper.[9] In this paper, we describe the fermentation, isolation procedures and some chemical and biological properties of FK-506. As shown in Fig. 1, FK-506 is a neutral macrolide. Determination of the chemical structure of FK-506 will be published elsewhere.[10]

Materials and Methods

Fermentation

A seed medium 100 ml containing glycerol 1%, corn starch 1%, glucose 0.5%, cotton seed meal 1%, corn steep liquor 0.5% and calcium carbonate 0.2% at pH 6.5 was poured into a 500-ml Erlenmeyer flask and sterilized at 120°C for 30 minutes. A loopful of slant culture of *S. tsukubaensis* No.

9993 was inoculated to the medium and cultured at 30°C for 4 days. The resultant culture was transferred to the same seed medium (20 liters) in 30 liters jar fermentor which had been sterilized at 120°C for 30 minutes in advance. After the culture was performed at 30°C for 2 days, aerated at 20 liters per minutes and agitated at 300 rpm, 16 liters of the preculture was inoculated to a fermentation medium 1,600 liters containing soluble starch 4.5%, corn steep liquor 1%, dried yeast 1%, calcium carbonate 0.1% and Adekanol (Asahi Denka Co.) 0.1% at pH 6.8 in a 2-kiloliter tank which had been sterilized at 120°C for 30 minutes in advance. This was cultured at 30°C for 4 days with agitation at 170 rpm and with air flow of 1,600 liters per minute.

Antimicrobial Activities

Minimum inhibitory concentration (MIC) test was conducted by the usual serial agar dilution method, using a nutrient agar for antibacterial test and a SABOURAUD's agar for antifungal test which was incubated at 30°C for 24 hours. MIC value is expressed as the minimum concentration which inhibits growth of the microorganisms.

Mice

Specific pathogen free BALB/C, C57BL/6, BDF_1, (female, 6~7 weeks old) and C3H/He (male, 6~7 weeks old) mice were obtained from Charles River Japan Inc. (Kanagawa, Japan).

Drugs

Prednisolone (PRD) was obtained from Sigma Chemical Co. (MO, U.S.A.). CS was a generous gift from Sandoz Ltd., Biological and Medical Research (Basel, Switzerland). For *in vitro* experiment, FK-506 and other drugs were dissolved in ethanol and further diluted in medium to the proper concentration in culture. For *in vivo* experiments, FK-506 and CS were dissolved in olive oil.

Suppression of Mixed Lymphocyte Reaction (MLR)

The MLR test was performed in microtiter plates, with each well containing 5×10^5 C57BL/6 responder cells (H-2^b), 5×10^5 mitomycin C treated (25 μg/ml mitomycin C at 37°C for 30 minutes and washed three times with RPMI 1640 medium) BALB/C stimulator cells (H-2^d) in 0.2 ml RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM sodium bicarbonate, benzylpenicillin (50 U/ml) and streptomycin (50 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2: 95% air for 68 hours and pulsed with [^{3}H]thymidine ([^{3}H]TdR) (0.5 μCi) 4 hours before the cells were collected. The test compounds were dissolved in ethanol at 1 mM and further diluted in RPMI 1640 medium and added to the cultures.

Plaque Forming Cell (PFC) Assay

PFC Assay: C3H/He mice were immunized on day 0 with 0.2 ml of 1×10^8 washed sheep erythrocytes intravenously. Spleens were removed on day 4 and spleen cells were incubated in the presence of sheep red blood cells as described by CUNNINGHAM and SZENBERG.[11] Tests were evaluated by enumeration of direct generated PFC's in the presence of complement. The number of spleen cells was counted with a Microcell counter CC-130 (Sysmex, Japan) and PFC results were calculated as PFC/10^6 recovered cells and PFC/spleen.

Drug Treatment: Drugs were administered orally for 4 days starting from the day of immunization.

Delayed Type Hypersensitivity Response to Methylated Bovine Serum Albumin (MBSA)

BDF_1 mice were sensitized with a subcutaneous injection of 0.1 ml emulsion consisting of equal volume of MBSA (2 mg/ml) and FREUND's incomplete adjuvant. Seven days later, a 0.05-ml challenge dose of 0.4 mg/ml MBSA in saline was injected into the plantar region of the right hind foot and 0.05 ml saline into the left hind foot to act as a control. Twenty four hours after challenge, both hind feet were measured with a dial gauge and the mean challenge in footpad thickness was measured. Drugs were injected for 8 days orally, starting from the day of sensitization.

Localized Graft-vs-host Reaction (GvHR)

Spleen cells prepared from C57BL/6 mice were slowly injected into the right footpad of BDF_1 mice as recipients at 10^7 cells/mouse. Seven days later, the recipients were sacrificed and popliteal

lymph nodes were removed from both extremities. The increase in lymph node weight due to the GvHR was measured as a difference between the popliteal lymph node weight of right (injected) leg and the popliteal lymph node weight of left (uninjected) leg. Drugs were administered orally for 5 days after injection of allogenic spleen cells.

Statistical Analysis

The results of each experiment were evaluated using Student's t-test.

Results

Fermentation

The time course of FK-506 production by *Streptomyces tsukubaensis* No. 9993 in a 2-kiloliter tank is shown in Fig. 2. The organism reached the stationary phase of growth after a 40-hour incubation. FK-506 production began at 40 hours, and the maximum accumulation was observed after a 90-hour incubation period. The pH of the medium during incubation fell to 6.2 and then began to rise gradually to 7.3 with the maximum accumulation of the compound.

Isolation Procedure

The flow diagram of the isolation procedure described below is shown in Fig. 3. The cultured broth (1,500 liters) was filtered with the aid of diatomaceous earth (25 kg). The mycelial cake was extracted with acetone (500 liters), yielding 500 liters of the extract. The acetone extract from the mycelium and the filtrate (1,350 liters) were combined and passed through a column of a non-ionic adsorption resin Diaion HP-20 (Mitsubishi Chemical Industries Ltd., Japan) (100 liters). After washing with water (300 liters) and 50% aqueous acetone (300 liters), elution was carried out with

Fig. 2. Time course of FK-506 production in a 2-kiloliter tank.
Potency of FK-506 was measured by MLR. PMV: Packed mycelium volume.

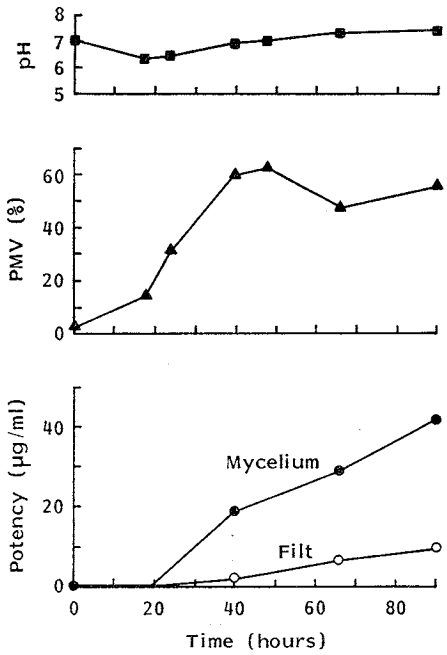
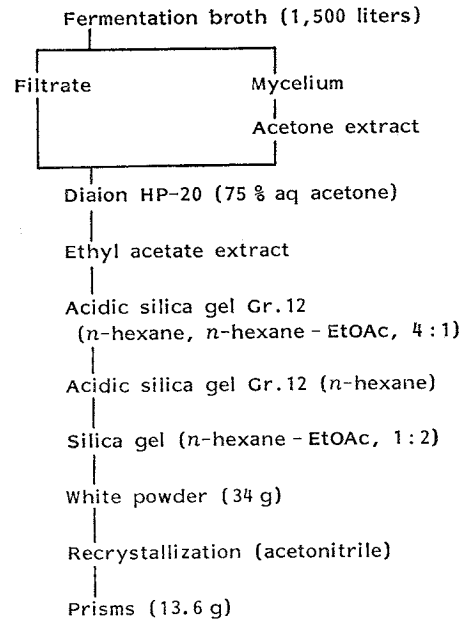


Fig. 3. Isolation procedure of FK-506.



75% aqueous acetone. The eluate was evaporated under reduced pressure to give residual aqueous solution (300 liters). This residue was extracted with ethyl acetate (20 liters) three times. The ethyl acetate extract was concentrated under reduced pressure to give an oily residue. The oily residue was mixed with twice its weight of acidic silica gel (special silica gel grade 12, Fuji Devison Co., Japan), and this mixture was slurried in ethyl acetate. After the solvent was evaporated, the resultant dry powder was subjected to column chromatography of the same acidic silica gel (8 liters) which was packed with *n*-hexane. The column was developed with *n*-hexane (30 liters), a mixture of *n*-hexane and ethyl acetate (4:1, 30 liters) and ethyl acetate (30 liters). The fractions containing FK-506 were collected and concentrated under reduced pressure to give an oily residue. The oily residue was mixed with twice its weight of acidic silica gel and this mixture was slurried in ethyl acetate. After the solvent was evaporated, the resultant dry powder was rechromatographed on acidic silica gel (3.5 liters) packed with *n*-hexane. The column was developed with *n*-hexane (10 liters), a mixture of *n*-hexane and ethyl acetate (4:1, 10 liters) and ethyl acetate (10 liters). Fractions containing FK-506 were collected and concentrated under reduced pressure to give an yellowish oil. The oily residue was dissolved in a mixture of *n*-hexane and ethyl acetate (1:1, 300 ml) and subjected to column chromatography of silica gel (230~400 mesh, Merck Co., Ltd., U.S.A.) (2 liters) packed with the same solvent system. Elution was carried out with a mixture of *n*-hexane and ethyl acetate (1:1, 10 liters and 1:2, 6 liters) and ethyl acetate (6 liters).

Fractions containing FK-506 were collected and concentrated under reduced pressure to give FK-506 in the form of white powder (34 g). This white powder was dissolved in acetonitrile and concentrated under reduced pressure. This concentrate was kept at 5°C overnight and prisms (22.7 g) were obtained. Recrystallization from the same solvent gave purified FK-506 (13.6 g) as colorless prisms.

Physico-chemical Properties

The physico-chemical properties of FK-506 are summarized in Table 1. It is soluble in methanol, ethanol, acetone, ethyl acetate, chloroform, diethyl ether, sparingly soluble in hexane, petroleum ether and insoluble in water. Color reactions are as follows: Positive in ceric sulfate, sulfuric acid, Ehrlich, Dragendorff and iodine vapor tests, negative in ferric chloride, ninhydrin and Molisch tests. Its Rf values on TLC are listed in Table 2. The determination of the chemical structure of FK-506 will be described elsewhere.[10]

Biological Properties

Antimicrobial Activities

The antimicrobial activities are shown in Table 3. FK-506 shows antifungal activity against

Table 1. Physico-chemical properties of FK-506.

Appearance	Prism
MP (°C)	127~129
Molecular formula	$C_{44}H_{69}NO_{12}\cdot H_2O$
Mass spectrum	804 (M+1, SI-MS)
Optical rotation	$[\alpha]_D^{23}$ −84.4° (*c* 1.02, $CHCl_3$)
Elemental analysis (%)	
Calcd for $C_{44}H_{69}NO_{12}\cdot H_2O$:	C 64.29, H 8.71, N 1.70.
Found:	C 64.20, H 8.86, N 1.72.
UV spectrum	End absorption
IR spectrum ($CHCl_3$) cm^{-1}	3530, 1750, 1730, 1710, 1650, 1100

SI-MS: Secondary ion mass spectrum.

Table 2. Rf values of FK-506 on silica gel plate.

TLC solvent system	Rf value
$CHCl_3$ - MeOH (10 : 1)	0.58
EtOAc	0.52

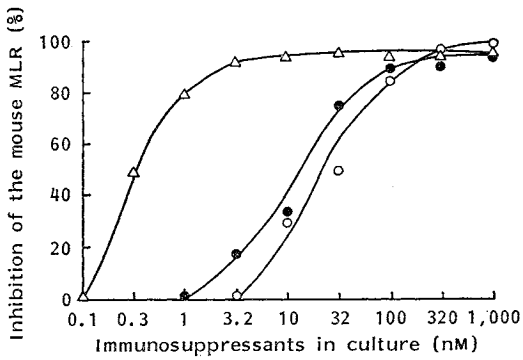
Table 3. Antimicrobial activity of FK-506.

Organisms	MIC (μg/ml)
Staphylococcus aureus	>100
Bacillus subtilis	>100
Proteus vulgaris	>100
Pseudomonas aeruginosa	>100
Escherichia coli	>100
Candida albicans	>100
Aspergillus fumigatus IFO 5840	0.025
Mucor hiemalis FZ-035	>100
Phialophora verrucosa FD-146	>100
Sporotrichum schenckii FD-158	>100
Fusarium oxysporum IFO 5942	0.05
Trichophyton asteroides	>100

Fig. 4. Effect of immunosuppressants on mouse MLR.

The data are presented as the percentage of inhibition based on response in the control diluent. Mean cpm of [^{3}H]TdR uptake for mouse MLR was 46,628±2,973. Unstimulated lymphocytes average 2,350 cpm of [^{3}H]TdR.

FK-506 (△), CS (○) and PRD (●) were added directly throughout the assay.



Aspergillus fumigatus and *Fusarium oxysporum*. It has no inhibitory effect on bacteria or yeast.

Suppression of MLR

Fig. 4 shows the suppressive effect of various concentrations of FK-506, CS and PRD on mouse MLR. The IC_{50} values (the concentration causing 50% inhibition) under present study conditions, were 0.32 nM, 27 nM and 17 nM, respectively. FK-506 shows much higher activity than CS and PRD on suppression of mouse MLR. Further details of *in vitro* immunosuppressive effect concerning FK-506 will be published in the succeeding paper.[12]

Suppressive Effect on Humoral and Cellular Immunity in Mice

As shown in Table 4, oral administration of FK-506 and CS strongly suppressed PFC response, but slightly decreased the number of spleen cells. Consequently both compounds strongly suppressed humoral immunity (antibody production) with marginal decrease in the number of spleen cells. The ED_{50} values (the dose causing 50% effect) of FK-506 and CS were 4.4 mg/kg and 39 mg/kg, respectively. FK-506 showed higher activity than CS on PFC response in mice.

As shown in Table 5, oral treatment with FK-506 and CS strongly suppressed the DTH response, therefore both compounds strongly suppressed cellular immunity. The ED_{50} values were 14 mg/kg and 40 mg/kg, respectively. FK-506 again showed higher activity than CS on the DTH response in mice.

Suppressive Effect on Localized GvHR in Mice

The effect of FK-506 and CS on the localized GvHR was examined by oral administration of both drugs to BDF_1 mice for 5 days after injection of spleen cells from C57BL/6 mice into the footpads. The results of GvHR were shown in Table 6. Treatment with FK-506 at doses of 10 mg/kg or more resulted in a significant weight reduction of popliteal lymph nodes from the injection site of allogenic cells. CS also inhibited GvHR, but it was less active on GvHR than was FK-506.

Table 4. Effect of FK-506 and CS on *in vivo* PFC response in mice.

Expt No.	Drug	Dose[a] (mg/kg)	*n*	PFC-spleen	No. of spleen cells $\times 10^6$	PFC/10^6 cells
I	Vehicle		5	316,800±26,700	63.6±5.8	5,054±408
	FK-506	3.2	5	209,600±37,300 (33.8)[b]	56.9±3.8	3,618±476 (28.2)
		10	5	59,800±10,200[c](81.1)	43.5±3.5	1,382±243[c](72.6)
		32	5	25,600± 2,200[c](91.9)	39.0±2.6[c]	657±41[c] (87.0)
		100	5	10,800± 1,600[c](96.6)	38.8±0.4[c]	278±41[c] (94.5)
II	Vehicle		5	297,000±27,000	69.7±8.1	4,388±408
	CS	10	5	283,000±29,000 (4.7)	65.6±3.4	4,335±439 (1.2)
		32	5	183,000±32,000 (38.4)	67.9±4.0	2,637±322[c](40.0)
		100	5	10,000± 3,000[c](96.6)	46.9±4.1	218±64[c](95.0)

[a] Drugs were dissolved in olive oil and administered orally.
[b] The number in parentheses represents the inhibition % of PFC.
[c] $P<0.01$ as compared with vehicle group.

Table 5. Effect of FK-506 and CS on DTH to MBSA in mice.

Expt No.	Drug	Dose[a] (mg/kg)	*n*	Footpad swelling ($\times 10^{-3}$ cm)
I	Vehicle		5	57.4±3.4
	FK-506	3.2	5	69.0±6.3 (−20.2)[b]
		10	5	31.0±4.4[c](46.0)
		32	5	20.8±4.4[c](63.8)
		100	5	7.8±1.6[c](86.4)
II	Vehicle		6	51.3±3.2
	CS	10	5	44.6±3.2 (13.1)
		32	5	26.8±5.2[c](47.6)
		100	5	11.8±1.8[c](77.0)

[a] Drugs were dissolved in olive oil and administered orally.
[b] The number in parentheses represents the inhibition % of DTH.
[c] $P<0.01$ as compared with vehicle group.

Acute Toxicity of FK-506 in Mice

The LD_{50} value of FK-506 when given intraperitoneally to BALB/C mice was greater than 200 mg/kg.

Discussion

CS is a metabolite extracted from a fungus and is a cyclic polypeptide consisting of 11 amino acids.[13] On the other hand, FK-506 is a metabolite extracted from a *Streptomyces* and is a neutral macrolide as shown in Fig. 1. So, the chemical structure of FK-506 is entirely different from that of CS. FK-506 inhibits the production of T cell-derived soluble mediators such as IL-2, IL-3 and IFN-γ as does CS. These *in vitro* actions of FK-506 will be published in the succeeding paper.[12] Fig. 4 shows the suppressive

Table 6. Effect of FK-506 and CS on GvHR in mice.

Expt No.	Drug	Dose[a] (mg/kg)	Mean enlargement of lymph nodes (mg)
I	Vehicle		7.1±0.7
	FK-506	3.2	5.2±0.5 (26.1)[b]
		10	3.9±0.8 (45.0)
		32	2.9±0.3[c](58.6)
		100	1.3±0.2[c](81.6)
II	Vehicle		6.5±0.2
	CS	10	6.0±0.5 (8.0)
		32	7.0±0.3 (−7.1)
		100	2.3±0.4[c](65.3)

[a] Drugs were dissolved in olive oil and administered orally.
[b] The number in parentheses represents the inhibition % on GvHR.
[c] $P<0.01$ as compared with vehicle group.

effect of FK-506 on mouse MLR, which has been thought to be the representative of IL-2 dependent T cell growth.[14] This compound has much higher potency than CS on mouse MLR. Furthermore, oral administration of FK-506 strongly suppressed both the production of antibody to SRBC (Table 4) and the DTH response to MBSA in mice (Table 5). It also inhibited GvHR induced by the injection of allogenic cells in mice (Table 6). These results clearly demonstrate that FK-506 is capable of inhibiting the humoral and cellular immunity and alloantigen-driven proliferation across strong histocompatibility barriers. Moreover these results suggest that FK-506 has much higher potency than CS not only *in vitro* but also *in vivo*. This leads us to conclude that FK-506 has profound immunosuppressive properties *in vitro* and *in vivo*, and it is one of the most active immunosuppressants. Further details of *in vivo* effects of the agent on the graft rejection in organ transplantation will be published in a separate paper.[15]

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