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

II. IMMUNOSUPPRESSIVE EFFECT OF FK-506 *IN VITRO*

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The immuno-pharmacological profile of a novel immunosuppressive agent, FK-506 produced by a streptomycete, is presented here. We proceeded to test the effect of the agent on various *in vitro* immune systems. It showed that mixed lymphocyte reaction, cytotoxic T cell generation, the production of T cell-derived soluble mediators such as interleukin 2 (IL-2), interleukin 3 and gamma-interferon and the expression of the IL-2 receptor were suppressed by this agent. The IC_{50} values of FK-506 and ciclosporin (CS) in all tests were approximately 0.1 nM and 10 nM, respectively. Therefore, the novel agent, FK-506 suppressed *in vitro* immune systems at about hundred times lower concentration than CS.

During the course of our research program for discovery of immunosuppressive substances in the natural products, we found a new immunosuppressant, FK-506 from the fermentation broth of a strain of *Streptomyces*. The chemical structure of this agent belongs to macrolides. It is water insoluble and soluble in alcohol. The immunosuppressive effect of this agent was more highly potent than ciclosporin (CS) *in vitro* and *in vivo*.[1]

CS, a fungal metabolite, is a powerful immunosuppressive agent that may represent one of the most important therapeutic advances in clinical organ transplantation in recent years.[2,3] By many investigator's results obtained from *in vitro* studies and animal models, at least two well established mechanisms have been advanced to explain the effects of CS; first, CS inhibits the release of lymphokines *in vitro*, especially interleukin 2 (IL-2);[4,5] secondly, CS therapy prevents clonal expansion of helper and cytotoxic T cells[6,7] and semi-selectively spares suppressor cells.[8,9]

In this paper, the immuno-pharmacological profile of the new immunosuppressive agent, FK-506, is presented in *in vitro* studies compared with CS.

Materials and Methods

Mice

Specific pathogen free (SPF) BALB/C and C57BL/6 mice (female, 6~7 weeks old) were obtained from Charles River Japan Inc. (Kanagawa, Japan). SPF C3H/HeJ mice (male, 6~7 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME, U.S.A.). They were maintained under SPF conditions until used.

Medium

RPMI 1640 medium, EAGLE's minimum essential medium (EMEM), DULBECCO's modified

EAGLE's medium (DMEM) and McCOY's medium 5A were purchased from Flow Laboratories (Rockville, MD, U.S.A.). Fetal calf serum (FCS) was purchased from Gibco (Gland Island, NY, U.S.A.). Unless otherwise indicated, complete medium comprised RPMI 1640, EMEM or DMEM, supplemented with 10% heat-inactivated FCS, 2×10^{-6} M sodium bicarbonate, 5×10^{-5} M 2-mercaptoethanol (2ME), 50 U/ml benzylpenicillin and 50 μg/ml streptomycin.

Drugs

FK-506, CS and prednisolone (PRD) were dissolved in ethanol at 1 mM and further diluted in medium and added to the cultures. CS was a generous gift from Sandoz Ltd., Biological and Medical Research (Basel, Switzerland). PRD was obtained from Sigma Chemical Co. (MO, U.S.A.).

Cells

C57BL/6 thymoma EL4, AKR/J thymoma BW 5147, DBA/2 mastocytoma P815 and human T cell leukemia Molt 4 were maintained in RPMI 1640 complete medium. L929, a murine fibrosarcoma cell line was maintained as an adherent line *in vitro* by serial passage in DMEM complete medium using trypsinization (trypsin 0.25%, Gibco). CTLL-2 cells, IL-2 dependent murine T cell line were maintained in DMEM complete medium supplemented with 20% AOFS supernatant stimulating with concanavalin A (Con A) (10 μg/ml). AOFS cells were maintained in DMEM complete medium. WEHI-3 cells, IL-3 producing cell line were maintained in RPMI 1640 complete medium. FDCP-2 cells, IL-3 dependent line were maintained in RPMI 1640 complete medium supplemented with 20% WEHI-3 supernatant.

Preparation of Spleen Cells

The spleen obtained from BALB/C and C57BL/6 mice was teased into single cell suspensions and filtered through nylon mesh. The cell suspension was freed of erythrocytes by treatment with ammonium chloride buffer (ACK buffer) and washed three times. Cell preparation was finally resuspended in RPMI 1640 complete medium.

Preparation of Peripheral Blood Lymphocytes (PBL)

Human PBL were obtained from healthy adult donors. The PBL were isolated by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) density centrifugation. The collected cells were washed three times and filtered through nylon mesh. Cell preparation was finally resuspended in RPMI 1640 complete medium.

Mouse Mixed Lymphocyte Reaction (MLR)

The mouse bulk MLR was performed in 24-well tissue culture plates (Corning Glass Works, Corning, NY, U.S.A.), with each well containing 5×10^6 C57BL/6 spleen cells (responder cells, H-2^b), 5×10^6 mitomycin C-treated (25 μg/ml mitomycin C at 37°C for 30 minutes and washed three times with RPMI 1640 medium) BALB/C spleen cells (stimulator cells, H-2^d) in 2 ml RPMI 1640 complete medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 - 95% air.

Human MLR

The human MLR test was established by using normal human PBL Responder lymphocytes (2.5×10^5 cells) were co-cultured with an equal number of mitomycin C treated stimulator lymphocytes in 0.2 ml RPMI 1640 complete medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 - 95% air for 96 hours. The cultures were pulsed with 0.5 μCi of [^{3}H]thymidine ([^{3}H]TdR, specific activity, 6.7 Ci/nmol; New England Nuclear, Boston, MA, U.S.A.) during the final 4 hours and harvested onto glass fiber filter paper with a micro-harvestor (Bellco. Glass Inc., Vineland, NJ, U.S.A.). The filter papers were dried and processed for liquid scintillation counting.

Human bulk MLR was performed in 24-well tissue culture plates in a volume of 2 ml/well. Therefore, 2.5×10^6 responding lymphocytes were co-cultured with an equal number of mitomycin C-treated stimulating lymphocytes in RPMI 1640 complete medium.

Cytotoxic Assay

The cytotoxicity tests, used EL4 thymoma, BW 5147 thymoma and Molt 4 lymphoma, were per-

formed in flat-bottom microtiter plates, with each well containing 10^4 cells in 0.1 ml RPMI 1640 complete medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 - 95% air for 72 hours. The cultures were pulsed with 0.5 μCi of [^{3}H]TdR during final 4 hours and counted.

Colony Forming Unit per Culture (cfu-C) Assay

The marrow plugs from C3H/HeJ mice were flushed out of femurs and dissociated by repeated aspiration until a single cell suspension was obtained. Bone marrow mononuclear cells (1.5×10^5) were plated into 6-well tissue culture plates (Nunclon, Denmark) in 1.0 ml of 0.45% Bacto agar (Difco Laboratories, Detroit, MI, U.S.A.) culture medium that included McCoy's medium 5A supplied with additional essential and nonessential amino acids, vitamins, glutamine, serine, asparagine, and sodium pyruvate, and contained 15% heat inactivated FCS and 10% L929 culture supernatants as colony-stimulating factors. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 - 95% air and scored for colonies after 7 days of incubation. The number of colonies on the plates was determined with a dissecting microscope. Aggregates of 40 or more cells were defined as colonies.

L929 Supernatants Preparation: L929 cells were grown as adherent monolayers in tissue culture flasks (F75, Corning) in 25 ml DMEM complete medium. Cells were seeded at 10^5 per flask, and the supernatants were collected and centrifuged 7 days later and filtered through a Millex 0.22 μm filter (Millipore Corp., Bedford, MA, U.S.A.).

IL-2 Assay

IL-2 activity was measured according to the method described by GILLIS and SMITH.[10] Briefly, the IL-2 dependent CTLL-2 cell line was used to quantitate IL-2 activity. CTLL-2 cells, 4×10^3, were cultured in flat-bottom microtiter plates at 37°C for 24 hours with various dilutions of IL-2 containing supernatants from bulk MLR (0, 24, 48, 72 and 96 hours) in 0.1 ml of DMEM complete medium. [^{3}H]TdR uptake was measured by pulsing cultures with 0.5 μCi of [^{3}H]TdR for 6 hours. The unit value was calculated by dilution analysis of test sample and was compared with a laboratory standard preparation in which 100 U is equivalent to the amount of IL-2 necessary to achieve 50% proliferation of CTLL-2 cells.

The laboratory standard preparation was supernatant from culturing AOFS cells (IL-2 producing hybridoma). AOFS cells (5×10^5/ml) were incubated with Con A (10 μg/ml) in tissue culture flasks (F75, Corning) in a volume of 30 ml DMEM complete medium for 24 hours, at which time the supernatants were harvested, centrifuged to remove the cells, and filtered through a filter (0.22 μm).

IL-3 Assay

The IL-3 dependent FDC-P2 cell line was used to quantitate IL-3 activity. FDC-P2 cells, 1×10^4, were cultured in flat-bottom microtiter plates at 37°C for 24 hours with various dilutions of IL-3 containing supernatants from bulk MLR (0, 24, 48, 72 and 96 hours) in 0.1 ml of RPMI 1640 complete medium. [^{3}H]TdR uptake was measured by pulsing cultures with 0.5 μCi of [^{3}H]TdR for 4 hours.

The unit value was calculated by dilution analysis of test samples and was compared with a laboratory standard preparation in which 100 U is equivalent to the amount of IL-3 necessary to achieve 50% proliferation of FDC-P2 cells. The laboratory standard preparation was supernatant from culturing WEHI-3 cells (IL-3 producing cell line). WEHI-3 cells (1×10^6/ml) were incubated in tissue culture flasks (F75, Corning) in a volume of 30 ml RPMI 1640 complete medium for 48 hours, at which time the supernatant were harvested, centrifuged to remove the cells, and filtered through a Millex 0.22 μm filter.

Gamma-interferon (IFN-γ) Assay

IFN-γ titers were quantitated by a conventional cytopathic effect reduction assay using L929 cells and vesicular stomatitis virus (VSV) as the challenge virus. The culture supernatants were serially diluted in flat-bottom microtiter plates in a volume of 50 μl/well. To this were seeded 50 μl of L-cells (4.5×10^5 cells/ml) in EMEM supplemented with 10% FCS. After 24 hours, the cultures were drained and the monolayers in each well were challenged with 50 μl of 100 $TCID_{50}$ (50% tissue culture-infective doses) of VSV in serum-free EMEM. About 24 hours later, when the control wells which

had not received IFN showed a complete cytopathic effect, the cells were stained with 50 μl of neutral red (125 μg/ml) in EMEM with 5% FCS for 90 minutes at 37°C. To quantitate the cytopathic effect, the dye was eluted with 50% ethanol in 0.1 M $NaH_2PO_4 \cdot 2H_2O$ (pH 4.5), according to the method of FINTER[11] with minor modifications. The absorbance of each well was read on a two-wavelength microplate photometer (Model MTP-22; Corona Electric Co., Ltd., Katsuta, Japan) at 550 nm. The percentage of dye uptake was calculated for each well relative to the average virus (0%) and average cell (100%) control. The 50% end points were determined and the sample titer were expressed in IU/ml in comparison with an international IFN standard.

Cell Mediated Lympholysis (CML) Assay

Effector Cells: Single cell suspensions of effector cells were obtained from the mouse bulk MLR (120 hours). The collected cells were washed three times and cellular debris was removed by passing through nylon mesh. They were finally resuspended in RPMI 1640 complete medium (−2ME).

Target Cells: Suspension culture of P815 mastocytoma (H-2^d) cells maintained in RPMI 1640 complete medium were washed twice and resuspended in serum free RPMI 1640 medium.

^{51}Cr Release Cytotoxic Assay: The test was performed in round-bottom microtiter plates (Linbro Chemical., New-Haven, CT, U.S.A.). P815 cells (4×10^6) in RPMI 1640 medium were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (specific activity 97 mCi/mg; JRIAm, Tokyo, Japan) for 60 minutes at 37°C. After washing three times, 1×10^4 target cells in 0.1 ml of RPMI 1640 complete medium (−2ME) were mixed with various concentrations of effector cells in 0.1 ml of complete medium (−2ME), to give effector to target ratios of 20:1, 10:1 and 5:1. After incubation for 4 hours at 37°C in CO_2 incubator, supernatants were carefully collected from each well by a Titertek automatic harvesting system (Flow Laboratories, Rockville, MD, U.S.A.) and counted in a gamma counter.

Supernatants from wells containing target cells alone in 0.2 ml medium served as controls for the baseline release of ^{51}Cr and 1×10^4 of ^{51}Cr-labeled target cells were also counted to determine total ^{51}Cr incorporated into the target cells.

The percentage of specific cytotoxicity was calculated as follows:

$$(\%)\text{Cytotoxicity} = \frac{\text{cpm of experimental group} - \text{cpm of medium control}}{\text{Total cpm} - \text{cpm of medium control}} \times 100$$

Lytic units were calculated according to the method of BRUNNER *et al.* by plotting % cytotoxicity versus the log of the number of effector cells. The number of effector cells required for 20% lysis (estimated by linear regression analysis) was arbitrarily defined as 1 lytic unit. Data are expressed as the number of lytic units per 10^7 primed lymphocytes.

Inhibition of Lymphocyte IL-2 Receptor Expression in Human MLR by FK-506

The human bulk MLR were established by using normal human PBL. FK-506 and CS were added to the human MLR at the initiation of the cultures. After 6 days the cells were harvested, washed and resuspended in RPMI 1640 complete medium.

Phosphate-buffered saline (PBS) containing 0.1% NaN_3 and 2% FCS was used as the staining buffer. The cultured cells were washed three times in this buffer, incubated in 20 μl of Fluorescein-isothiocyanate (FITC)-conjugated CD25 (anti-Tac monoclonal antibody) solution (0.1 mg/ml antibody in this buffer) at 4°C for 30 minutes, then the cells were washed three times and analyzed on a fluorescence-activated cell sorter (FACS Analyzer, Becton Dickinson. Mountain View, CA, U.S.A.). For all direct immunofluorescence sample, 1×10^4 live cells determined by the exclusion of propidium iodide (PI) were analyzed. This permits highly sensitive and reliable FITC/PI one color immunofluorescence.

Determination of the Percent of Positive Cells: Six day MLR cells generated in the presence of immunosuppressants were incubated with CD25 or fluorescence control IgG1 (FITC). Fluorescence intensity profiles of experimental and control cells, normalized for total cell number, were superimposed, and the cross-over point was estimated. Areas under the curves to the right of the cross-over were determined and expressed as a percentage of total area. Control percentage area was subtracted from experimental yielding an estimate of the percent of positive cells.

Statistical Analysis

Results were analyzed for statistical significance by the Student's t-test.

Results

Suppression of Human MLR by FK-506

In our previous paper, we described the suppressive effect on mouse MLR by FK-506, CS and PRD. The IC_{50} values were 0.32 nM, 27 nM and 17 nM respectively.[1]

FK-506, CS and PRD were tested for its ability to suppress the *in vitro* proliferative response of human lymphocytes to alloantigen stimulation. The results of representative experiments in which various quantities of FK-506 and CS were added to the human MLR at the initiation of culture are illustrated in Fig. 1. The IC_{50} values of FK-506, CS and PRD were 0.22 nM, 14 nM and 80 nM, respectively.

Effect of FK-506 on Constitutive T Cell Proliferation

Various dilutions of FK-506, CS and PRD were added to the cultures of mouse thymoma EL4, BM 5147, and human T-leukemia Molt 4 cell lines. After 3 days incubation, the amount of incorporated [^{3}H]TdR was measured. A typical and representative dose-response curve of FK-506 and CS is presented in Fig. 2. The percent inhibition of FK-506 at 3,200 nM in these three assays was 24.1%, 8.7% and 15.3%, so the IC_{50} values were more than 3,200 nM. On the other hand, the IC_{50} values of CS were 2,300 nM, 1,100 nM and more than 3,200 nM, respectively. The IC_{50} values of PRD were more than 3,200 nM, 32 nM and more than 3,200 nM, respectively (data not shown). PRD strongly inhibited the growth of BW 5147 cells.

This results suggest that FK-506 was non-toxic at less than 3,200 nM against constitutive murine and human T cell proliferation such as EL4, BW 5147 and Molt 4 cell lines.

Fig. 1. Effect of FK-506, CS and PRD on human MLR.

The data are presented as the percentage of inhibition based on response in the control diluent. FK-506 (△), CS (○) and PRD (●) were added throughout the assay. Mean cpm of [^{3}H]TdR uptake for human MLR was 11,000±735. Unstimulated lymphocytes average 506 cpm of [^{3}H]-TdR.

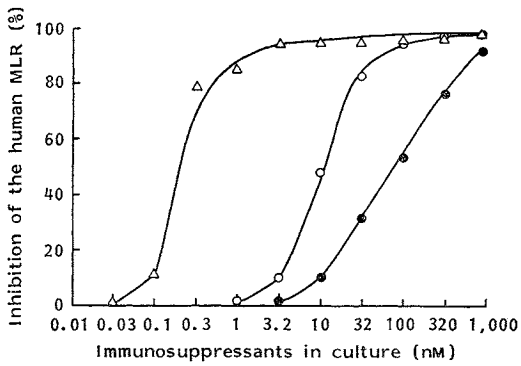
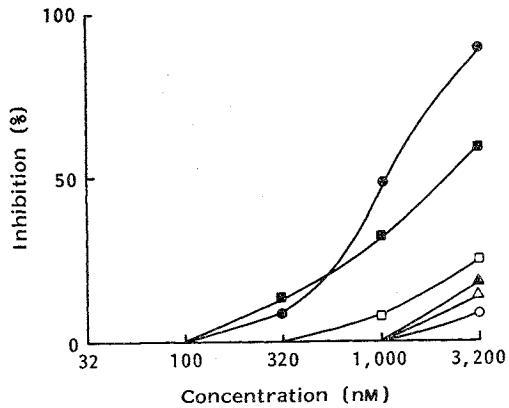


Fig. 2. Effect of FK-506 and CS on proliferation of mouse and human T leukemia.

Each point represents the inhibition % based on response in the control diluent. Mean cpm of [^{3}H]TdR uptake for EL4, BW 5147 and Molt 4 were 27,706±568, 284,304±5,553 and 9,753±349, respectively.

BW 5147; +FK-506 (○), +CS (●): EL4; +FK-506 (□), +CS (■): Molt 4; +FK-506 (△), +CS (▲). Drugs were added throughout the assay.



Effect of FK-506 on cfu-C

The effect of FK-506, CS and PRD on the formation of granulocyte/macrophage colonies by murine bone marrow cells cultured with colony stimulating factors (CSF, L929 supernatants) in semi-solid agar is shown in Fig. 3. The IC_{50} values of FK-506, CS and PRD were 1,400 nM, 800 nM and 140 nM, respectively. The growth and maturation of myeloid progenitor cells *in vitro* depends upon CSF, therefore FK-506 was non-toxic against myeloid progenitor cells approximately less than 1,000 nM.

Suppression of FK-506 on the Generation of Cytolytic T Lymphocytes (CTL)

Alloreactive CTL were generated from bulk MLR by mixing 5×10^6 C57BL/6 mouse lymphocytes with 5×10^6 mitomycin C-treated BALB/C mouse lymphocytes. Various dilutions of FK-506, CS and PRD were added to mouse bulk MLR at the initiation of culture. After a 5-day incubation, cells were washed and the cytotoxicity of the CTL was determined by a short-term ^{51}Cr-release micro-cytotoxicity assay by using P815 cells as targets. As demonstrated in Table 1, FK-506 and CS significantly decreased the induction of CTL in a dose-dependent manner. The IC_{50} values of FK-506 and CS were 0.2 nM and 24 nM, respectively. PRD had negligible inhibitory on CTL generation and rather caused some enhancement at a high dose.

A similar suppressive effect of FK-506 and CS was observed in a different allogeneic system in which BALB/C mouse lymphocytes were co-

Fig. 3. Effect of FK-506, CS and PRD on mouse cfu-C.

The data are expressed as inhibition % based on response in the control diluent. Mean number of colonies/culture was 90.0±2.7 in control group. FK-506 (△), CS (○) and PRD (●) were added at the initiation of the culture.

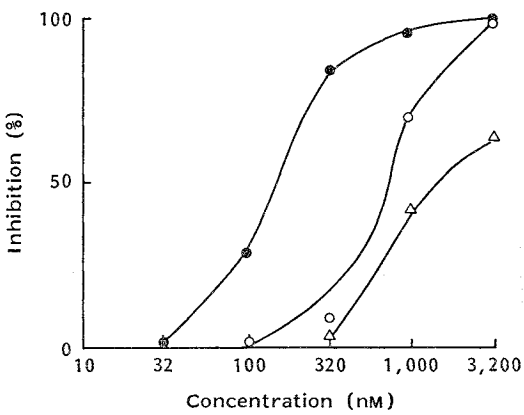


Table 1. Effect of FK-506 and CS on CTL generation.

Immunosuppressants (nM)		Specific lysis (%) E/T 20:1	10:1	5:1	Lytic unit (% inhibition)
Control	0	49.6	35.2	25.8	270 (0)
FK-506	10	7.6	4.4	2.8	1.4 (99.5)
	1	18.0	10.9	4.4	35.7 (86.8)
	0.3	15.1	11.2	5.6	26.3 (90.3)
	0.1	46.8	32.8	26.7	286 (—)
CS	100	10.6	6.6	3.7	10.4 (96.1)
	32	22.7	19.3	10.5	74.1 (72.6)
	10	46.0	33.3	25.0	256 (5.2)
	3.2	48.7	39.4	27.0	333 (—)
PRD	320	60.7	51.1	43.5	1,190 (—)
	100	76.3	62.7	56.6	2,500 (—)
	32	37.3	27.7	19.9	182 (52.6)
	10	23.7	17.2	9.9	71 (73.7)
	3.2	23.0	14.9	8.8	167 (38.1)

Table 2. Effect of FK-506 and CS on IL-2 production of supernatants from mouse MLR.

Immunosuppressants (nM)		IL-2 production; U/ml (% inhibition)		
		Day 1	Day 2	Day 3
Control	0	8.0 (0)	30.9 (0)	13.5 (0)
FK-506	10	0 (100)	0 (100)	0 (100)
	1	0 (100)	0 (100)	0 (100)
	0.3	0.1 (98.8)	0.6 (98.1)	0.7 (94.8)
	0.1	4.0 (50)	15.9 (48.5)	7.5 (44.4)
CS	100	0 (100)	0.5 (98.4)	0 (100)
	32	0.3 (96.3)	6.1 (80.3)	4.9 (63.7)
	10	0.9 (88.8)	20.6 (33.3)	8.7 (35.6)
	3.2	6.8 (15)	25.9 (16.2)	9.6 (28.9)

Table 3. Effect of FK-506 and CS on IL-2 production of supernatants from human MLR.

Immunosuppressants (nM)		IL-2 production; U/ml (% inhibition)			
		Day 1	Day 2	Day 3	Day 4
Control	0	2.0 (0)	7.2 (0)	8.5 (0)	1.9 (0)
FK-506	10	0 (100)	0 (100)	0 (100)	0 (100)
	1	0 (100)	0 (100)	0 (100)	0 (100)
	0.3	0.04 (98.0)	0.7 (90.3)	0.2 (97.6)	0 (100)
	0.1	0.7 (65.0)	4.5 (37.5)	3.3 (61.2)	0.1 (94.7)
CS	100	0 (100)	0 (100)	0 (100)	0 (100)
	32	0 (100)	0.06 (99.2)	0.1 (98.8)	0.02 (98.9)
	10	0 (100)	0.2 (97.2)	0.3 (96.5)	0.1 (94.7)
	3.2	1.3 (35.0)	6.9 (4.2)	6.0 (29.4)	1.3 (31.6)

Table 4. Effect of FK-506 and CS on IL-3 production of supernatants from mouse MLR.

Immunosuppressants (nM)		IL-3 production: U/ml (% inhibition)			
		Day 1	Day 2	Day 3	Day 4
Control	0	1.7 (0)	37.0 (0)	41.3 (0)	52.5 (0)
FK-506	10	0 (100)	0 (100)	0.1 (99)	2.6 (95)
	1	0 (100)	0.3 (99)	4.6 (89)	8.7 (83)
	0.3	0 (100)	10.3 (73)	21.3 (48)	22.6 (57)
	0.1	1.1 (35.3)	18.8 (50)	39.7 (4)	35.0 (33)
CS	100	0 (100)	0 (100)	1.8 (96)	10.0 (81)
	32	0 (100)	11.1 (71)	44.7 (—)	45.9 (13)
	10	1.1 (35)	22.9 (39)	51.9 (—)	45.0 (14)
	3.2	0.6 (65)	23.3 (38)	48.0 (—)	72.7 (—)

cultured with mitomycin C-treated C57BL/6 mouse lymphocytes for 5 days and the cytotoxicity of these effector cells was tested by using EL4 as targets.

Up to this point, it has been shown that the new agent, FK-506, suppressed the *in vitro* proliferative response of lymphocytes to alloantigen stimulation and cytotoxic T cell generation at about hundred times lower concentration than did CS and was shown that this agent was as non-toxic as CS. Then, we investigated the effect of FK-506 on the production of T cell-derived soluble mediators, such as IL-2, IL-3 and IFN-γ induced by alloantigen.

Suppression of IL-2 Production from Mouse and Human MLR by FK-506

We investigated the effect of non-toxic concentrations of FK-506 and CS on the production of

Table 5. Effect of FK-506 and CS on IFN-γ production of supernatants from mouse MLR.

Immunosuppressants (nM)		IFN-γ production; IU/ml (% inhibition)			
		Day 1	Day 2	Day 3	Day 4
Control	0	47.8 (0)	253.8 (0)	440.5 (0)	202.2 (0)
FK-506	10		14.9 (94.1)	23.0 (94.8)	14.6 (92.8)
	1		11.9 (95.3)	20.8 (95.3)	22.0 (89.1)
	0.3		13.1 (94.8)	71.8 (83.7)	27.3 (86.5)
	0.1		162.5 (36.0)	294.0 (33.3)	136.1 (32.7)
CS	100		10.0 (96.1)	14.0 (96.8)	13.0 (93.6)
	32		18.2 (92.8)	32.5 (92.6)	25.0 (87.6)
	10		54.5 (78.5)	74.3 (83.1)	63.1 (68.8)
	3.2		124.1 (51.1)	265.7 (39.7)	135.8 (32.8)

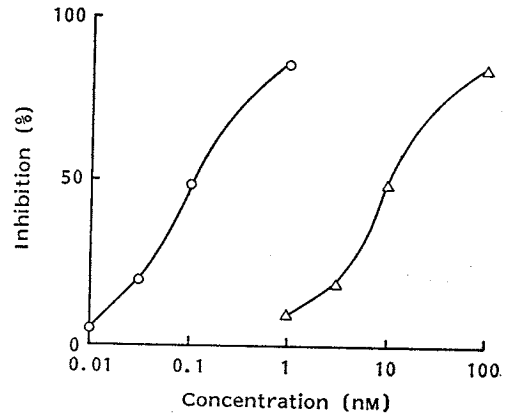
IL-2. To determine whether FK-506 inhibited the production of IL-2 in MLR supernatants, graded quantities of FK-506 were added to bulk MLR at the initiation of culture and supernatants were tested for IL-2 activity, using the IL-2 dependent CTLL-2 cells line. The representative data are shown in Table 2 for mouse experiments and Table 3 for human experiments. In both experiments IC_{50} values of FK-506 and CS were approximately 0.1 nM and 10 nM, respectively.

Suppression of IL-3 Production from Mouse MLR by FK-506

In the same way, we tested the supernatants of mouse bulk MLR for IL-3 activity using the IL-3 dependent FDC-P2 cell line. The results of representative experiment are shown in Table 4. IC_{50} values of FK-506 and CS were approximately 0.3 nM and 32 nM, respectively.

Fig. 4. Suppression of IL-2 receptor expression in human MLR.

The IL-2 receptor expression in human MLR was determined by the percent Tac positive cells using FACS Analyzer. The data are presented inhibition % based on response in the control diluent. Each point is the mean of four individual experiments. FK-506 (○) and CS (△) were added at the initiation of bulk MLR.



Suppression of IFN-γ Production from Mouse MLR by FK-506

In the same way, we tested the supernatants of mouse bulk MLR for IFN-γ activity using the dye uptake method of L929-VSV system. The results of representative experiment are shown in Table 5. IC_{50} values of FK-506 and CS in suppression of IFN-γ production from mouse MLR were approximately 0.1 nM and 3.2 nM, respectively.

Suppression of IL-2 Receptor Expression in Human MLR by FK-506

The 6-day human MLRs were established by using normal human PBL. Graded dilutions of FK-506 or CS were added to the bulk lymphocytes at the initiation of the culture. Cultivated live cells determined by the exclusion of PI were then analyzed on a FACS Analyzer. IL-2 receptor expression in the human MLR was determined by the percent of Tac positive cells. As shown in Fig. 4, FK-506 and CS inhibited the expression of IL-2 receptor on lymphocytes. The IC_{50} values of FK-506 and CS were approximately 0.1 nM and 10 nM, respectively.

Discussion

CS has been used successfully as the primary drug to suppress the rejection of transplants.[2,3] However, its specific immunosuppressive effect on the one hand, and clinical nephrotoxicity on the other, have led us to search for more specific and less toxic new immunosuppressants. FK-506 was discovered in 1984 in our laboratories during a search for immunosuppressive substances among natural products. This agent was isolated from the fermentation broth of a strain of *Streptomyces*.[1]

The data obtained from the present experiments show that the new immunosuppressant, FK-506, strongly inhibited the proliferative response of lymphocytes to alloantigen stimulation, the cytotoxic T cell generation, the expression of IL-2 receptor and the production of T cell derived soluble mediators such as IL-2, IL-3 and IFN-γ at non-toxic concentrations. Further, it was shown that the new agent suppressed at about hundred times lower concentration than did CS in all tests. The toxic concentration of this agent against T lymphoma is more than 10^{-6} M (Figs. 2 and 3) and the effective concentration of immunosuppression is about 10^{-10} M. Meanwhile, the former of CS is more than 10^{-6} M and the latter is about 10^{-8} M. The immunosuppressive effect of FK-506 has a high degree of selectivity.

The MLR and the assay of CTL used here are generally regarded *in vitro* correlates of allograft rejection.[12] FK-506 inhibited both mouse and human MLR. FK-506 also inhibited mouse CTL generation. Accordingly, FK-506 is an effective immunosuppressant that may prove useful in organ transplantation.

Glucocorticosteroids exert complex, and generally suppressive effects on lymphocytes and on immune responses. In particular, it has been found that glucocorticosteroids inhibit mitogen- or antigen-induced T cell proliferation.[6] In our experiments, PRD suppressed antigen induced mouse and human T cell proliferation. (Fig. 1) However, PRD strongly inhibited BW 5147 cell's growth and cfu-C. (Figs. 2 and 3) The immunosuppressive effect of PRD is less specific than FK-506 and CS. The suppression on cfu-C of PRD may cause to depress myelopoiesis and erythropoiesis, meanwhile, FK-506 and CS may not. Furthermore, PRD had negligible effects on CTL generation and enhanced at high dose. (Table 1)

The mode of action of FK-506 and CS is different from that of PRD and seems to be more specific suppression on the proliferative response of lymphocytes to alloantigen stimulation, the cytotoxic T cell generation. The immunosuppressive effects of CS may be based, at least in part, on its ability to inhibit production of the lymphokines by T lymphocytes, especially IL-2 or expression of IL-2 receptor.[4,5] FK-506 also suppressed at about hundred times lower concentration than CS on the production of lymphokines and the expression of IL-2 receptor. Though the cascade and the action of lymphokines are still unclear, it may prevent the clonal expansion of T cells and the cytotoxic T cell generation with the inhibition on the production of lymphokines by T lymphocytes, especially IL-2 or the expression of IL-2 receptor.

In certain conditions, IL-2 is required for the production of IFN-γ,[13] and IFN-γ, in turn, induces the expression of IL-2 receptors.[14] These lymphokines are also involved in differentiation and activation of cytotoxic effects of CML,[15] so they are likely to play a role in organ transplant rejection and acceptance. IL-3 can promote the growth of certain lymphocytes that are not IL-2 dependent, and it may be a suppressor cell growth, activation, or maturation factor.[16] FK-506 and CS required slightly higher concentration than IL-2 and IFN-γ to suppress the production of IL-3 (Table 4), so FK-506 and CS therapy may semi-selectively spare suppressor cells. Though the mode of action of FK-506 remains unclear, it should provide a valuable tool for investigating the immunological actions of lymphokines.

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