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Tacrolimus-Rapamycin Combination Therapy for Experimental Autoimmune Uveoretinitis

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Abstract: Tacrolimus and rapamycin both belong to a new family of immunosuppressants, immunophilin ligands, but the mechanisms by which they inhibit T cell activation are different. Therefore, we tested the immunosuppressive effects of combination therapy with low doses of tacrolimus and rapamycin on experimental autoimmune uveoretinitis (EAU) in rats. Male Lewis rats, immunized with S-antigen (S-Ag) were given intraperitoneal injection of the combined drugs for 14 days after the immunization with S-Ag. Effects were evaluated by clinical observations, histological examination and immune response. The combination therapy with tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 mg/kg per day) achieved 100% suppression of clinical EAU and 66.7% suppression of histological EAU; tacrolimus combined with a higher dose of rapamycin (0.1 mg/kg per day) caused 100% suppression clinically and histologically. Therapy with either drug alone achieved only partial suppression: tacrolimus alone (0.1 or 0.2 mg/kg per day) or rapamycin alone (0.03–0.2 mg/kg per day). Doubling the dose of either drug produced only 16.7% suppression with rapamycin or 50% suppression with tacrolimus. The serum antibody levels to S-Ag and proliferative response of lymphocytes to S-Ag were also significantly suppressed by the combination therapy with low doses of tacrolimus and rapamycin. **Jpn J Ophthalmol 1997;41:396–402** © 1997 Japanese Ophthalmological Society

Key Words: Experimental autoimmune uveoretinitis, rapamycin, rat, tacrolimus.

Introduction

Tacrolimus (FK506) is a new antibiotic of the macrolide family isolated from the fermentation broth of *Streptomyces tsukubaensis*.[1] The compound binds to a specific cytosolic binding protein, FK-binding protein (FKBP), forming a complex which inhibits expression of the early phase of T-cell activation genes, thus inhibiting T-cell-mediated immune responses. The effect of tacrolimus on uveitis was tested in retinal soluble antigen (S-Ag)-induced experimental autoimmune uveoretinitis (EAU); it suppressed the development of EAU at doses 30 times lower than cyclosporine, when given from the day of immunization.[2] Furthermore, tacrolimus suppressed the intensity of EAU when given only after the onset. The efficacy of tacrolimus and its safety for uveitis patients were tested in a multi-center clinical trial in Japan. The clinical study revealed that tacrolimus was effective in refractory uveitis including Behçet's disease, although there were a variety of adverse side effects which included renal impairment, neurological symptoms and hyperglycemia.[3] Rapamycin, also an antibiotic of the macrolide family, was isolated from the fermentation broth of *Streptomyces hydroscopicus* more than a decade ago.[4–5] Although the structure of rapamycin is very similar to tacrolimus (Figure 1) and the two compounds share the same cytosolic binding protein (FKBP), their molecular mechanisms of immunosuppression are different. Tacrolimus inhibits the early phase of T-cell activation genes, while rapamycin inhibits the late phase of IL-2 receptor-mediated T-cell activation. Rapamycin inhibited development of S-Ag-induced EAU at doses between 0.025 and 1.0 mg/kg per day when administered by continuous intravenous infusion by miniosmotic pump.[6]

Because the therapeutic applications of cyclosporine, tacrolimus, and rapamycin appear to be very limited, combination therapy with these drugs at low

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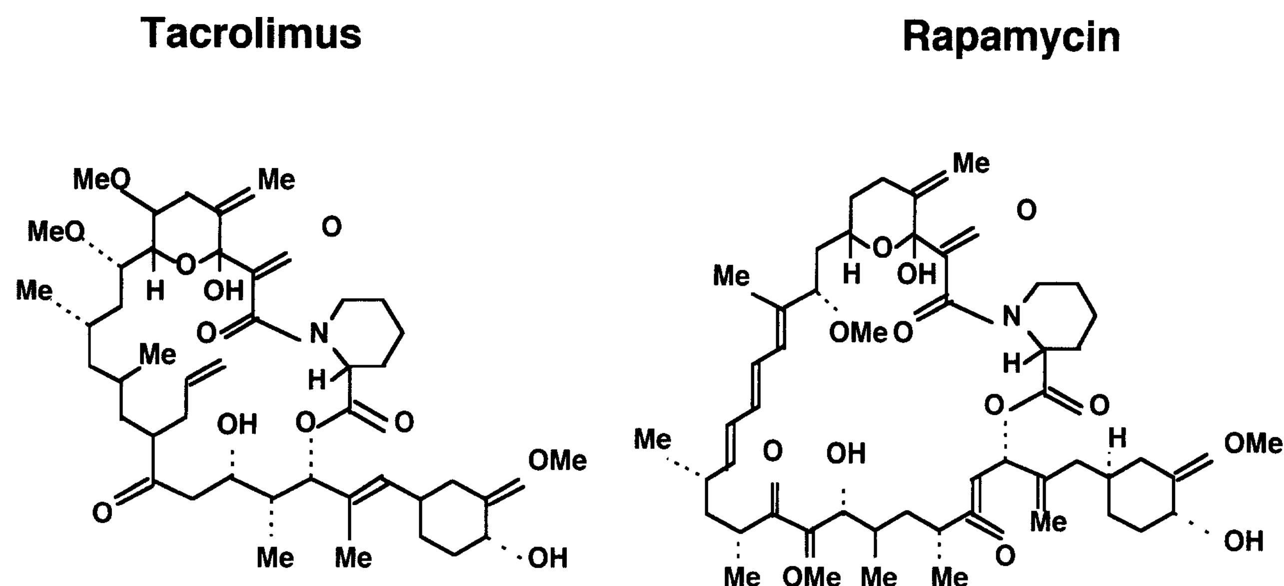

Figure 1. Structure of tacrolimus and rapamycin.

doses might be beneficial. Rapamycin (0.01 mg/kg per day) combined with cyclosporine (2 mg/kg per day) was significantly more effective in suppressing EAU than either drug alone.[7] The present study was, therefore, aimed at investigating the efficacy of combination therapy with tacrolimus and rapamycin for EAU.

Materials and Methods

Animals

Rats of the inbred Lewis strain were purchased from Charles River Japan (Atsugi, Kanagawa). Male rats 8–10 weeks of age were used. These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Immunization

S-Ag was prepared at our laboratory from bovine retinas according to the methods of Dorey et al[8] and Fujino et al.[9] The antigen was emulsified (1:1, v:v) in complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA), containing *Mycobacterium tuberculosis* H37 RA (Difco) at a concentration of 2.0 mg/mL. A total volume of 100 μL per rat, containing 20 μg S-Ag, was injected into one hind footpad.

Drugs

Tacrolimus (a gift from Fujisawa Pharmaceutical Co., Ltd., Osaka) was suspended in 1/15 mol/L phosphate-buffered saline (PBS) (pH 7.4), and rapamycin (a gift from Wyeth-Ayerst Research, Princeton, NJ, USA) was solubilized in a vehicle composed of 0.2% sodium carboxymethylcellulose (Sigma Chemical, St. Louis, MO, USA), 0.25% polyoxyethylene (20) sorbitan monooleate (Wako Pure Chemical Industries Ltd., Osaka), and distilled water.

Therapy

The rats were divided into three groups: single drug therapy with tacrolimus (0.1 or 0.2 mg/kg per day), single drug therapy with rapamycin (0.03, 0.06, 0.1, or 0.2 mg/kg per day), and combination therapy with tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 or 0.1 mg/kg per day). Drugs were given by intraperitoneal injection once a day on days 0–14 after immunization with S-Ag. Control rats were treated with intraperitoneal injection of PBS and the vehicle for rapamycin.

Evaluation of EAU

Rats were monitored daily under an operation microscope for clinical evaluation of EAU development. The onset of EAU was confirmed when fibrins were detected in the anterior chamber. The clinical score was graded into four categories: 0, no inflammation; 1+, mild inflammation with small fibrins in the anterior chamber; 2+, moderate inflammation with hypopyon; 3+, severe inflammation with hypopyon, hyphema, and protrusion of the eyeball.[10]

All eyes were enucleated under general anesthesia

with diethylether on days 15–17 after immunization, fixed in 2.5% glutaraldehyde 4% formaldehyde solution, and embedded in glycol methacrylate. Sections cut at 4 μm were stained with hematoxylin and eosin, and examined with a light microscope. The EAU scores by histological examinations were as follows: 0, no inflammation; 0.5+, inflammatory cell infiltration of the retina without lesion or with photoreceptor damage covering less than 1/4 of the retina; 1+, photoreceptor outer segment damage in ≥1/4 of the retina; 2+, lesion extending to the outer nuclear layer and in ≥1/4 of the retina; 3+, lesion extending to the inner nuclear layer and in ≥1/4 of the retina; 4+, full thickness retinal damage in ≥1/4 of the retina.[11]

Evaluation of the Immune Responses

Antibody levels in sera. The heparinized peripheral blood and spleen were collected under general anesthesia, before the rats were sacrificed by CO_2. Blood samples were used to measure the serum levels of antibody to S-Ag using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well polyvinylchloride plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) were coated with S-Ag (100 μL per well at a concentration of 5 μg/mL) in 1/15 mol/L phosphate buffer (PBS, Iatron Laboratories, Tokyo) at pH 9.6. After overnight incubation at 4°C and washing five times with 1/15 mol/L PBS (pH 7.4), the wells were incubated with 1/15 mol/L PBS (pH 7.4) containing 10% fetal calf serum (FCS) (Bioserum, Victoria, Australia) for 1 hour at room temperature. After washing the wells again as described above, an aliquot (100 μL) of serial dilution of serum samples, diluted with 1/15 mol/L PBS (pH 7.4) containing 10% FCS were added. Following incubation for 1 hour at room temperature, the wells were washed five times with 1/15 mol/L PBS (pH 7.4). One hundred μL of a 1:1000 dilution of peroxydase conjugated anti-rat IgG (Wako) was added to each well, and the plates were incubated for 1 hour at room temperature. Excess conjugates were washed out and 100 μL of substrate solution (0.4 mg/mL *o*-phenylenediamine in citrate PO_4 buffer, pH 4.8, supplemented with 0.0093% H_2O_2 shortly before use) was added to the wells. After 10 minutes of incubation at room temperature, the optical density (OD) was measured at 492 nm using an automated device (Titertek Multiscan MCC/340, Flow laboratories, McLean, VA, USA). Antibody levels were expressed as the absorbance values at 1:640 dilution of each serum sample.

Proliferative responses of lymphocytes. Proliferative responses of lymphocytes to S-Ag or Concanavalin A (Con A) (Sigma) were measured using non-adherent spleen cells. Briefly, the spleens from the rats were gently teased in RPMI-1640 medium (Gibco, NY, USA), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 2% FCS. The cell suspension was washed twice in the medium and was depleted of erythrocytes using 10% Tris-ammonium chloride. The cell suspension was incubated in 100 × 20 mm plastic dishes (Corning, NY, USA) at 4°C for 1 hour and nonadherent cells were collected. The nonadherent splenocytes were cultured in triplicate in a 96-well u-bottomed plate (4 × 10^5 cells/well) (Becton Dickinson Labware) in RPMI-1640 medium with 5% FCS. The cultures were stimulated with S-Ag (2 μg/mL) or Con A (5 μg/mL) for 4 days at 37°C with 100% humidity with 5% CO_2 in air, and pulsed with 1.0 μCi [H^3]thymidine for the last 16 hours of culture. The cells were harvested with an automatic cell harvester (Micro 96 harvester 11055, Skatron, Oslo, Norway), and incorporated radioactivity was measured by a liquid scintillation counter (LSC-1000, Aloka, Tokyo). The results were expressed as the arithmetic means of counts per minute (cpm) ± *SD* of triplicate measuring and the arithmetic means of stimulation index (cpm of stimulated cultures) / (cpm of nonstimulated cultures) ± *SD*.

Systemic Condition

To monitor the systemic condition of the rats, their body weight was measured before the therapy, on day 7 and day 14 during the drug therapy. The body weight was expressed as a percent increase of the baseline (before therapy).

Statistical Analysis

Statistical analysis was carried out using Student's *t*-test, χ^2 test, or Mann–Whitney *U*-test, as indicated.

Results

Effects of Tacrolimus and Rapamycin on Clinical EAU

The clinical evaluation of EAU in rats treated with tacrolimus and/or rapamycin is summarized in Table 1. All control rats treated with PBS and the vehicle (Group A in Table 1) developed severe EAU on day 11.8 ± 1.0 (mean ± *SD*) post-immunization. Single drug therapy with tacrolimus or rapamycin (Groups B–G) suppressed EAU in a dose-dependent

Table 1. Effects of Drug Therapy on Clinical EAU Development

	Drug (mg/kg per day)		EAU Development	
Group	Tacrolimus	Rapamycin	Eyes With EAU / Total	Clinical Score[a]
A	—	—	20 / 20[b]	2.4 ± 0.7
B	0.1	—	8 / 10[b]	1.1 ± 0.4
C	0.2	—	6 / 12[b]	2.7 ± 0.5
D	—	0.03	11 / 12[b]	2.5 ± 0.5
E	—	0.06	10 / 12[b]	2.6 ± 0.5
F	—	0.1	6 / 12[b]	2.2 ± 0.8
G	—	0.2	5 / 12[b,c]	1.4 ± 0.5
H	0.1	0.03	0 / 18	—
I	0.1	0.1	0 / 14	—

[a]Final clinical score of EAU eyes (mean ± *SD*).
[b]$P < 0.05$ by χ^2 test as compared with group H.
[c]$P < 0.05$ by χ^2 test as compared with group I.

manner. The lower dose of tacrolimus (0.1 mg/kg per day) had minimal effects and a twofold higher dose suppressed EAU in only one-half of the treated rats. Similarly, low doses of rapamycin (0.03 and 0.06 mg/kg per day) had minimal effects on EAU. Even with the highest dose of rapamycin tested, 0.2 mg/kg per day, a complete suppression of EAU was not achieved. In contrast to the single drug therapy, combination therapy with low doses of tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 or 0.1 mg/kg per day) achieved complete suppression of EAU by clinical evaluation and the difference was statistically significant (Table 1).

Drug Effects on Histological EAU

Drug effects were further evaluated by histological examinations (Table 2 and Figure 2). All eyes in

Table 2. Effects of Drug Therapy on Histological Score of EAU

	Drug (mg/kg per day)		Histological Examination	
Group	Tacrolimus	Rapamycin	Eyes With EAU / Total	Score[a]
A	—	—	20 / 20[b,c]	2.9 ± 1.3[d]
B	0.1	—	9 / 10[b,c]	1.6 ± 0.5[d]
C	0.2	—	6 / 12[c]	2.3 ± 1.4[d]
D	—	0.03	11 / 12[b,c]	3.1 ± 0.8[d]
E	—	0.06	10 / 12[b,c]	3.3 ± 0.9[d]
F	—	0.1	7 / 12[c]	2.0 ± 1.4
G	—	0.2	6 / 12[c]	2.3 ± 1.4[d]
H	0.1	0.03	6 / 18	1.0 ± 0.0
I	0.1	0.1	0 / 14	—

[a]Severity indicated by histological score of EAU eyes (mean ± *SD*).
[b]$P < 0.05$ by χ^2 test as compared with group H.
[c]$P < 0.05$ by χ^2 test as compared with group I.
[d]$P < 0.05$ by Mann Whitney U-test as compared with group H.

control rats (Group A in Table 2) exhibited high histological scores (Figure 2A). The histological examinations essentially confirmed the clinical evaluations. The highest doses of either tacrolimus (0.2 mg/kg per day) or rapamycin (0.2 mg/kg per day) alone caused only 50% suppression. Much higher suppression (12/18, 66.7%) was achieved by combination therapy (0.1 mg/kg per day of tacrolimus and 0.03 mg/kg per day of rapamycin), and the histological score in this group was significantly lower than control (Table 2 and Figure 2B). None of the eyes in rats treated with tacrolimus (0.1 mg/kg per day) combined with rapamycin (0.1 mg/kg per day) (Group I) developed EAU which could be detected by histological examinations (Figure 2C).

Drug Effects on Immune Responses

The antibody levels in the sera and proliferative responses of lymphocytes are summarized in Tables 3 and 4. Control rats (Group A) exhibited high levels of antibody to S-Ag, and high proliferative responses to S-Ag and Con A. A low dose of tacrolimus (0.1 mg/kg per day) did not suppress the antibody levels and proliferative responses of lymphocytes. Rapamycin (0.03 mg/kg per day) significantly suppressed only the antibody levels. A higher dose of rapamycin (0.1 mg/kg per day) suppressed both antibody levels and proliferative responses of lymphocytes. A combination therapy of tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 or 0.1 mg/kg per day) caused much stronger suppression in the antibody levels and proliferative responses of lymphocytes. In addition, the immune responses in the combination therapy groups were significantly lower than those in the group treated with tacrolimus alone (Group B).

Systemic Condition

Body weight was monitored as a parameter of the systemic toxic effects of the drugs (Figure 3). Control rats immunized with S-Ag without drug therapy gained body weight as time passed. None of the therapies tested in the study affected body weight compared to the control group.

Discussion

The data recorded here demonstrated that tacrolimus and rapamycin are either additive or synergistic in the suppression of EAU in rats. Combination therapy with low doses of tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 mg/kg per day) (Group H

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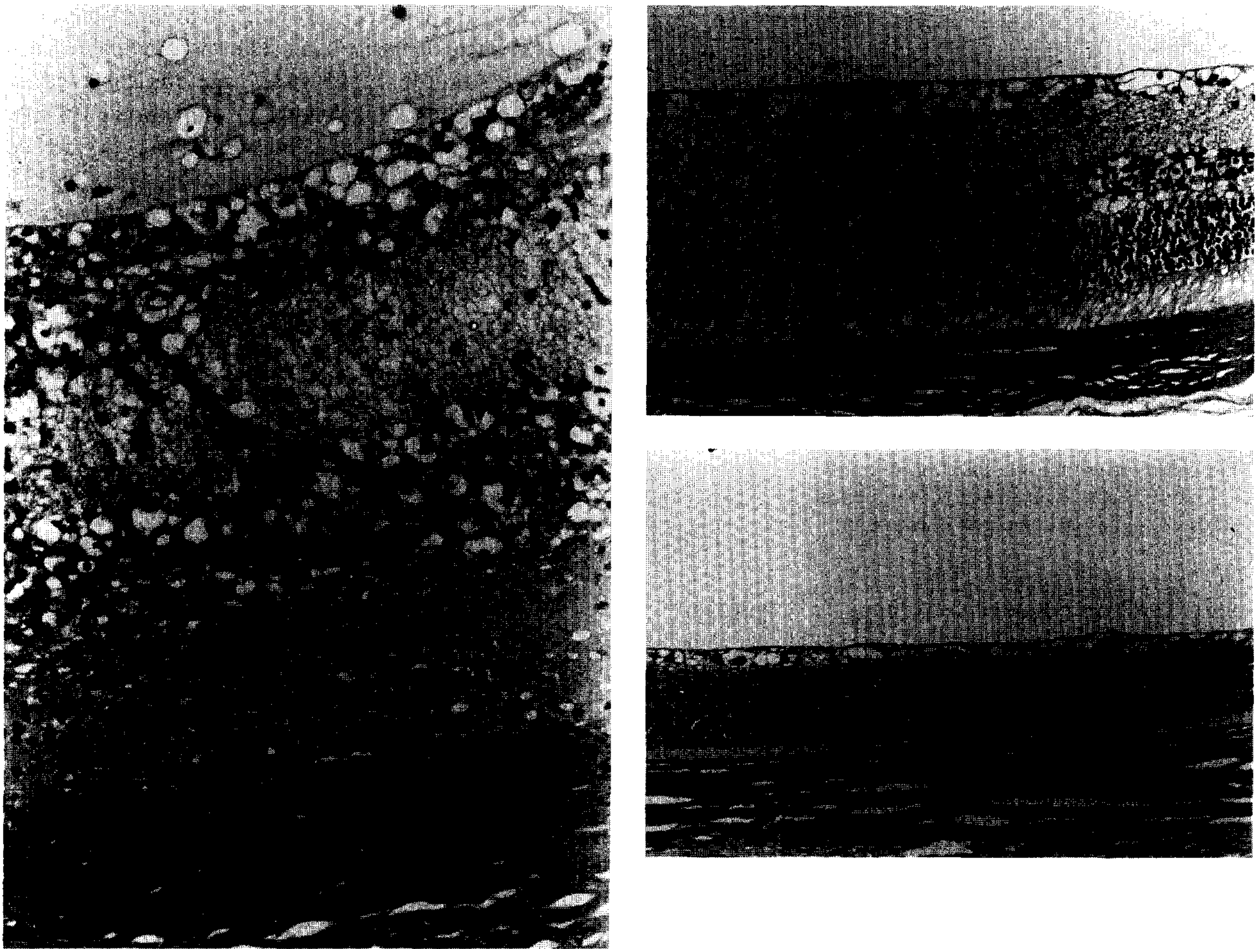

Figure 2. Histological changes in drug-treated rats (Hematoxylin and eosin, bar: 20 μm). (**A**) Control rat treated only with PBS and rapamycin vehicle: maximum (+4) EAU changes. (**B**) Rat treated with a combination of tacrolimus (0.1 mg/kg per day) and rapamycin (0.03 mg/kg per day): minimum (+1) EAU changes. (**C**) Rat treated with tacrolimus (0.1 mg/kg per day) and rapamycin (0.1 mg/kg per day) showing no change in EAU.

in Table 1) produced 100% suppression of clinical EAU, whereas doubling the dose of tacrolimus alone (0.2 mg/kg per day) or rapamycin alone (0.06 mg/kg per day) caused only 50% or 16.7% suppression, respectively (Groups C and E). Although one-third of the animals in the group undergoing combination therapy had EAU histologically (Group H in Table 2), its incidence and histological score was significantly lower than in animals in the single drug therapy groups. In addition, combination therapy with 0.1 mg/kg per day tacrolimus and 0.1 mg/kg per day rapamycin (Group I) caused a complete suppression of EAU by histological confirmation. In our previous study, a complete suppression of EAU was achieved by intraperitoneal tacrolimus at a dose of 1.0 mg/kg per day, which is a tenfold higher dose than used for combination therapy in the present experiment. These data strongly suggest that tacrolimus and rapamycin are synergistic, rather than additive. This notion was further supported by the drug effects on the immune responses.

Table 3. Effects of Drug Therapy on Antibody to S-Antigen

Group	Drug (mg/kg per day) Tacrolimus	Rapamycin	Antibody to S-Ag (OD at 492 nm)
A	—	—	1.281 ± 0.420 (n = 8)
B	0.1	—	1.149 ± 0.548 (n = 8)
D	—	0.03	0.531 ± 0.424 (n = 9)[a]
F	—	0.1	0.290 ± 0.345 (n = 9)[a]
H	0.1	0.03	0.162 ± 0.125 (n = 9)[a,b]
I	0.1	0.1	0.047 ± 0.017 (n = 11)[a,b]

[a] $P < 0.05$ by Student's t-test as compared with group A.
[b] $P < 0.05$ by Student's t-test as compared with group B.

Table 4. Effects of Drug Therapy on Lymphocyte Proliferation

	Drug (mg/kg per day)		Proliferative Response of Lymphocytes		
Group	Tacrolimus	Rapamycin	None	Con A (2 μg/mL)	S-Antigen (5 μg/mL)
A	—	—	4034.4 ± 1497.9[a] (*n* = 7)	107340.9 ± 81794.7 [26.6 ± 20.3]	10976.2 ± 6917.1 [2.7 ± 1.7][b]
B	0.1	—	3049.8 ± 910.9 (*n* = 6)	63827.1 ± 48589.9 [20.9 ± 15.9]	7551.7 ± 2493.0 [2.5 ± 0.8]
D	—	0.03	6070.6 ± 3099.2 (*n* = 6)	125100.8 ± 79005.0 [20.6 ± 13.0]	11501.4 ± 11711.0 [1.9 ± 1.9]
F	—	0.1	6390.5 ± 2400.0 (*n* = 5)	104210.5 ± 93985.0 [16.3 ± 14.7]	7733.6 ± 6212.3[c] [1.2 ± 1.0]
H	0.1	0.03	6474.7 ± 1719.9 (*n* = 7)	76868.5 ± 35339.5[c,d] [11.9 ± 5.5]	6497.0 ± 3723.9[c,d] [1.0 ± 0.6]
I	0.1	0.1	6157.6 ± 4028.2 (*n* = 5)	78271.0 ± 36584.7[c] [12.7 ± 5.9]	7614.3 ± 4327.3[c,d] [1.2 ± 0.7]

[a]Mean c.p.m. of incorporated [H^3] thymidine ± *SD*.
[b]Mean stimulation index ± *SD*.
[c]$P < 0.05$ by Student's *t*-test as compared with group A.
[d]$P < 0.05$ by Student's *t*-test as compared with group B.

Tacrolimus inhibits the expression of the early phase of T cell activation genes, including IL-2, IL-3, IL-4, gamma interferon, tumor necrotizing factor and granulocyte-macrophage colony stimulating factor. On the other hand, rapamycin has no effect on the expression of these early T cell activation genes, but strongly inhibits the late IL-2 receptor associated signal pathway. The differences in the immunosuppressive properties between the two drugs are considered to be, at least in part, attributed to the synergistic effects.

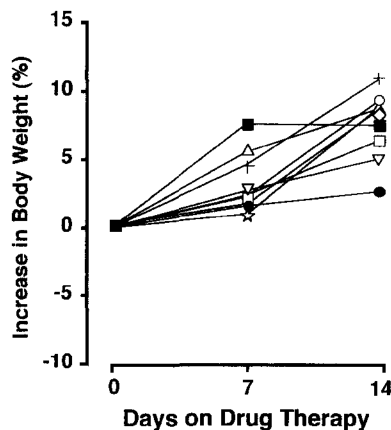

Figure 3. Body weight of drug-treated rats. The body weight was expressed as percent increase of body weight before treatment. +: control. □: tacrolimus 0.1 mg/kg per day. ☆: tacrolimus 0.2 mg/kg per day. △: rapamycin 0.03 mg/kg per day. ◇: rapamycin 0.06 mg/kg per day. ▽: rapamycin 0.1 mg/kg per day. ○: rapamycin 0.2 mg/kg per day. ■: tacrolimus 0.1 mg/kg per day + rapamycin 0.03 mg/kg per day. ●: tacrolimus 0.1 mg/kg per day + rapamycin 0.03 mg/kg per day.

The combination of the two drugs was well tolerated in the experiment. All rats gained body weight during therapy. Minimal weight loss occurred compared with the control group, but the body weight in the combination group was not statistically different from that in the control group. The most significantly toxicity of rapamycin reported in the literature was myocardial toxicity in the rat at 1.0 mg/kg per day[12] which is a thirtyfold higher dose than the dose used in this study. As for tacrolimus, a previous clinical study in uveitis patients revealed that therapeutic doses of tacrolimus by oral administration caused a variety of adverse side effects, such as renal impairment, neurological symptoms, gastro-intestinal symptoms and hyperglycemia.[3] Although the route of administration and species were different, 0.1 mg/kg per day of tacrolimus in combination with rapamycin (0.03 mg/kg per day) was not toxic, but effective in suppressing EAU in the present experiment. Therefore, the use of rapamycin in combination with tacrolimus allows administration of a dose of each drug much lower than the minimal toxic dose, and yet still maintains a therapeutic effect.

In conclusion, the present data suggest that a combination therapy with low doses of tacrolimus and rapamycin might be beneficial in treating patients with refractory uveitis, for better efficacy and less adverse side effects.

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