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Tacrolimus versus cyclosporin A: a comparative study on rat renal allograft survival

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Abstract Although tacrolimus has been studied in a wide variety of experimental animal models, we are the first group to systematically study the effect of tacrolimus on rat renal allograft survival, as primary therapy and as anti-rejection therapy, in comparison with cyclosporin A (CyA). Renal grafts were transplanted from BN to LEW rats. Tacrolimus and CyA were administrated orally from day 0 for 50 days as primary therapy after grafting. Allografts were rejected after a median survival time (MST) of 8 days. Both tacrolimus und CyA significantly prolonged renal allograft survival, in a dose-dependent manner compared with the allograft controls. The most effective dose was 3.2 mg/kg, per day for tacrolimus, and 10 mg/kg per day for CyA. There was no significant difference in renal function between the group treated with the most effective dose of tacrolimus and the CyA-treated group. The percentage of detectable serum IL-2 level was 45% in the allograft control group, but was undetectable in groups treated with the most effective dose of tacrolimus or CyA at days 3 and 6 after grafting. On the other hand, no side effects were noted in recipient rats by daily inspection, body weight change, and histological studies, although minimal tubular vacuolation was encountered in the group treated with CyA 32 mg/kg per day. In addition, the most effective doses of tacrolimus and CyA were studied as anti-rejection therapy. All of the 5 recipients treated with tacrolimus from days 2–14, and 3 of the 5 treated from days 4–16 after grafting, survived for more than 50 days. However, the MST was 19 days for recipients treated with CyA from days 2–14, and 13 days for those treated from days 4–16 after grafting. In summary, tacrolimus as primary therapy induced rat renal allograft survival with renal function and side effects comparable with those of CyA. Interestingly, when both agents were used as anti-rejection therapy, tacrolimus, but not CyA, could significantly overcome ongoing renal allograft rejection in the rat.

Key words Immunosuppression, Tacrolimus, cyclosporin A, Renal transplantation, Graft survival

Introduction

Tacrolimus, a macrolide antibiotic produced by *Streptomyces tsukubaensis* [14], is a strong immunosuppressive agent that is used successfully in clinical organ transplantation [2, 30]. It has been estimated to be three to ten orders of magnitude more potent than cyclosporin A (CyA), when used as a primary or as an anti-rejection immunosuppressive agent in liver transplantation [1, 30, 34, 35]. Initial results from clinical renal trans-

plantation studies using tacrolimus either as primary therapy or anti-rejection therapy have also been encouraging. Clearly, the immunosuppressive effect of this agent is greater than that of CyA. It appears that tacrolimus is at least as effective as CyA in clinical renal transplantation, and that in certain aspects (such as rejection frequency, dose requirement, incidence of hypertension, and overcoming ongoing rejection) tacrolimus may be superior to CyA [2, 10, 15]. In addition, tacrolimus has also been investigated in a wide variety of experimental animal models. Here the most consistent finding is its immunosuppressive activity, which is 3–100 times stronger than that of CyA, especially for skin, heart, liver, kidney, and small bowel allograft transplantation [6, 22, 23, 24, 33]. While the immunosuppressive effect of tacrolimus in experimental renal transplantation has been studied in several animal models, such as dogs, pigs, and nonhuman primates, its effect on renal allograft survival in the rat remains unexplored.

In the present study, the experiments were therefore designed to evaluate the effect of tacrolimus on rat renal allograft survival in comparison with CyA, and to show whether its effect is closely related to the result of clinical renal transplantation.

Materials and Methods

Animals

Male LEW ($RT1^l$ $RT2^b$) rats weighing 250–350 g were used as recipients. Male BN ($RT1^n$ $RT2^a$) rats, weighing 150–250 g were used as kidney donors. The animals were obtained from commercial sources (LEW: Charles River, Japan, and BN: Seiwa, Japan) and kept under specific-pathogen-free conditions in our animal facility.

Immunosuppressive therapy

Tacrolimus, an oral formulation, was suspended in water. CyA was dissolved in pure olive oil. A placebo, pure olive oil and vehicle, were used as the controls. Both agents were administered orally at a volume of 5 ml/kg per day.

Renal transplantation

Renal transplants were performed LEW and BN to LEW rats, using the modified technique described by Fisher and Lee [3]. Kidneys were transplanted with end-to-side anastomoses of the renal artery and vein with patches of aorta and inferior vena cava, respectively. The donor ureter was directly implanted into the recipient bladder over a fine polyethylene internal stent. All recipients were bilaterally nephrectomized at the time of transplantation. The graft ischemic time was approximately 30 min. Survival of kidney transplant was measured as time of rat survival. Graft rejection was confirmed by histological examination.

Blood sampling

Blood was drawn from the tip of the rats' tails. Whole blood tacrolimus and CyA trough levels were measured by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), respectively. Plasma creatinine was tested by CRE-EN kit (Kainos, Japan).

IL-2 bioassay

IL-2 activity in serum from recipient rats was measured according to the method described by Gillis and Smith [4]. Briefly, the IL-2 dependent CTLL-2 cell line was used to quantify IL-2 activity. CTLL-2 cells, $4X10^3$ were cultured in flat-bottom microtiter plates at 37 °C for 48 hours with various dilutions of IL-2 containing serum from LEW recipient rats in 0.2 ml of RPMI 1640 complete medium. [^{3}H]TdR uptake was measured by pulsing cultures with 0.5 μCi of [^{3}H]TdR during the final 6 hours. Cells were harvested onto glass fiber paper with an automatic harvester, and [^{3}H]TdR incorporation was measured in bate scintillation counter. The IL-2 levels in the serum sample were determined, based on a standard curve, that was constructed using a recombinant IL-2. The IL-2 level over the count of the serum sample from naive control rats was considered detectable.

Histological examination

For light microscopy, samples of kidney tissues from recipients were fixed in 10% neutrally buffered formalin and stained with hematoxylin and eosin.

Experimental design

Two separate experiments were performed. Nine groups ($n = 7$) were involved in experiment I: tacrolimus or CyA, as primary therapy, was orally administered from day 0 to day 50 after renal grafting (Table 1). Recipients were weighed every day during the study. Tacrolimus and CyA blood levels, and plasma creatinine levels were tested at days 3, 6, 10 after transplantation, and once a week thereafter. Serum IL-2 levels were measured at days 3 and 6 after grafting. Five groups ($n = 5$) were involved in experiment II: the most effective dose of tacrolimus or CyA from the experiment I was administered as anti-rejection therapy for 13 days, starting from day 2 or day 4 after grafting (Table 2). All kidney tissues obtained from both experiment I and experiment II after rat death were used for histological study.

Statistical analysis

The significance of the differences in days of renal graft survival between the experimental and the control groups, and in creatinine and body weight change between the most effective dose of tacrolimus and CyA treated groups was determined using the Kaplan-Meier test and student's t-test, respectively. A P value of < 0.05 was considered to reflect a significant difference.

Table 1 Survival of BN rat renal allografts in LEW recipient rats treated with tacrolimus from day 0 to day 50 posttransplantation, in comparison with cyclosporin A or CyA

Group	Drug	*n*	Survival days (*n*)[a]	Median time	*P* value
Isograft	(–)	15	> 100	> 100	
Allograft	Distilled water	7	6 (2), 7, 8 (2), 11, 15	8	
	Placebo	7	7 (2), 9 (5)	9	NS[bc]
	Olive oil	7	6 (2), 8 (2), 9, 13, 14	8	NS
	Tacrolimus 0.32 mg/kg	7	6, 7, 8, 10, 12, 17, 58	10	NS[d]
	Tacrolimus 1.0 mg/kg	7	17 (2), 22, 23, 28, > 100 (2)	23	< 0.01
	Tacrolimus 3.2 mg/kg	7	39, 63, > 100 (5)	> 100	< 0.01
	CyA 3.2 mg/kg	7	9, 11, 14 (2), 15, 16, > 100	14	< 0.05[e]
	CyA 10 mg/kg	7	78, > 100 (6)	> 100	< 0.01
	CyA 32 mg/kg	7	18, 25, 32, 57, 76, 79, > 100	57	< 0.01

[a] Numbers in parentheses indicate the numbers of individuals surviving for that amount of days

[b] NS = Nonsignificant

[c] Median comparison between the distilled water treated control and the placebo or the olive oil control group

[d] Median comparison between the tacrolimus treated groups and the placebo control

[e] Median comparison between the CyA treated groups and the olive oil control

Results

We studied the effects of tacrolimus and CyA as primary therapy on rat renal allograft survival. The results are shown in Table 1. Isografts were not rejected, and survived for more than 100 days. The median survival time (MST) of the allograft controls was 8 days when treated with water, 9 days when treated with placebo, and 8 days when treated with olive oil. Both tacrolimus and CyA significantly prolonged the life of renal allografts in a dose-dependent manner, compared to controls. The most effective dose for renal allograft survival was 3.2 mg/kg per day for tacrolimus, and 10 mg/kg per day for CyA, which induced the survival of 5 out of 7, and 6 out of 7 allografts respectively, for more than 100 days. Body weight was slightly lower in the group treated with the most effective dose of tacrolimus, as compared to that of the CyA-treated group, especially at day 50 post-transplantation, when a significant difference between the groups became apparent, but both groups maintained their improved condition throughout treatment (Fig. 1). There was no significant body weight increase in the group receiving CyA 32 mg/kg per day as compared with the baseline. Tacrolimus blood levels higher than 1 ng/ml and CyA levels higher than 200 ng/ml were considered therapeutic. Tacrolimus blood through levels around 2 ng/ml and CyA levels around 500 ng/ml could induce rat renal allograft survival inde-

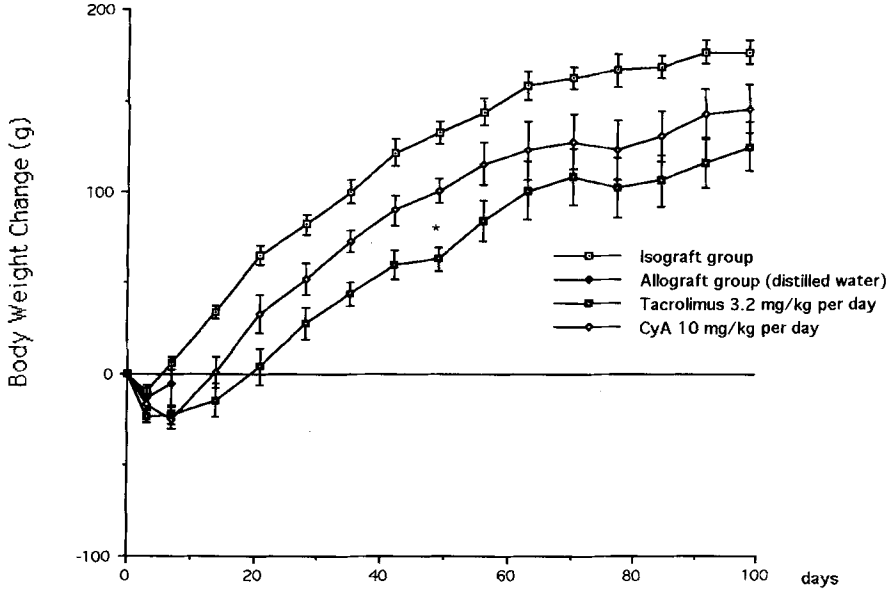

Fig. 1 Mean body weight changes in LEW recipient rats with BN rat renal grafts after treatment with tacrolimus (3.2 mg/kg/day) and CyA (10 mg/kg/day) compared with the controls. *$P < 0.05$

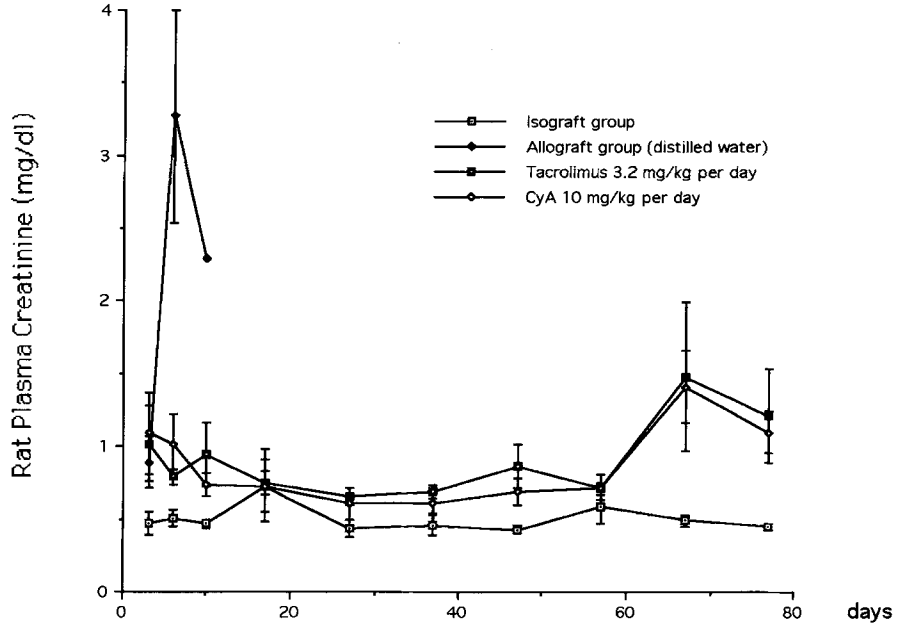

Fig. 2 Mean plasma creatinine levels in LEW recipient rats with BN rat renal grafts after tacrolimus (3.2 mg/kg/day) and CyA (10 mg/kg/day) treatment compared with the control groups

finitely. Plasma creatinine levels were closely correlated with renal graft function in our studies. Plasma creatinine increased rapidly in the allograft control and drug treated groups before rat death. In order to compare the renal function of the recipient rats treated with tacrolimus with that of those treated with CyA, we studied the kinetics of plasma creatinine levels in the groups receiving the most effective dose of tacrolimus and CyA. There was no significant difference between these two groups regarding plasma creatinine levels (Fig. 2). Although IL-2 serum levels in recipient rats were not consistently correlated with renal allograft rejection, the percentage of animals with IL-2 levels above that of naive control rat samples was significantly different between the allograft control and the drug treated groups. The percentage of recipient rats with IL-2 levels above that of the naive controls was 45% in allograft controls compared to 0% in both groups treated with the most effective dose of tacrolimus and CyA at day 3 and day 6 after grafting. The histopathological studies revealed severe interstitial hemorrhage, tubular necrosis, venous endothelialitis, and diffuse lymphoplasmacytic infiltration in the allograft controls (Fig. 3b) compared with the isograft controls (Fig. 3a), whereas rats treated with tacrolimus 3.2 mg/kg per day (Fig. 3c) or CyA 10 mg/kg per day (Fig. 3d) had only focal lymphoplasmacytic infiltration. On the other hand, no evidence of nephrotoxicity was noted in the histological study, although minimal tubular vacuolation occurred in the CyA 32 mg/kg per day treated group (Fig. 3e).

We further studied the most effective dose of tacrolimus (3.2 mg/kg per day) and CyA (10 mg/kg per day) as anti-rejection therapy. Tacrolimus and CyA were administered to 4 groups for 13 days starting from day 2 or day 4 after grafting. The MST for each group is shown in Table 2. Untreated allograft controls were rejected at day 8. All of the 5 recipient rats treated with tacrolimus at 3.2 mg/kg per day from day 2–14 and 3 of the 5

Table 2 Survival of BN rat renal allografts in LEW recipient rats after a short treatment with tacrolimus or cyclosporin A or CyA starting from day 2 or day 4 posttransplantation

Group	Drug	Period	*n*	Survival days (*n*)[a]	Median time	*P* value
Allograft	(–)		5	6, 7, 8, 10, 14	8	
1	Tacrolimus 3.2 mg/kg	2–14	5	> 50 (5)	> 50	< 0.01[b]
2	Tacrolimus 3.2 mg/kg	4–16	5	11, 28, > 50 (3)	> 50	< 0.01
3	CyA 10 mg/kg	2–14	5	7, 14, 19, 28, 50	19	< 0.05
4	CyA 10 mg/kg	4–16	5	6, 8, 13, 16, 21	13	NS[c]

[a] Numbers in parentheses indicate the numbers of individuals surviving for that amount of days

[b] Median comparison between the drug-treated group and the allogeneic control

[c] NS = Nonsignificant

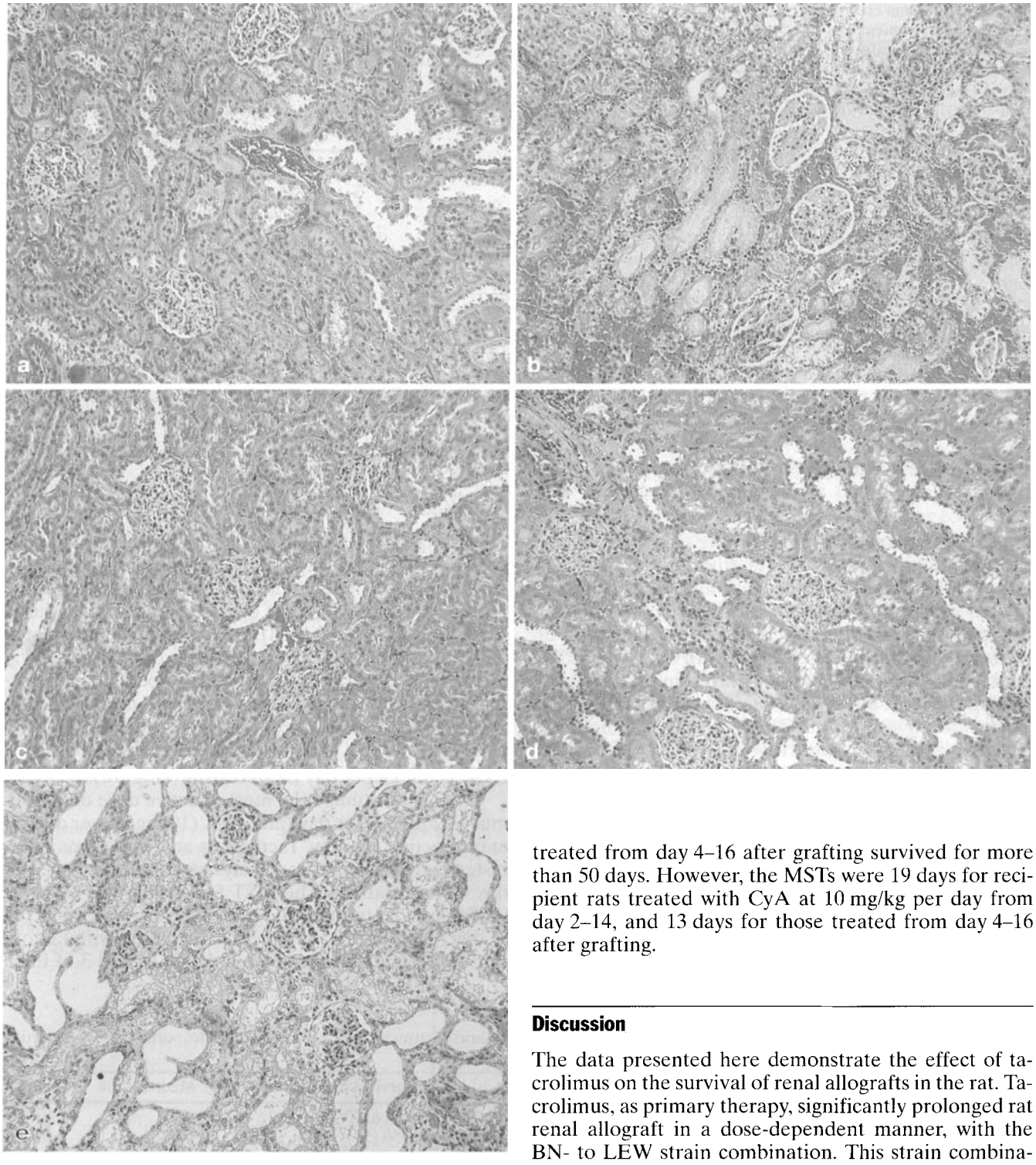

Fig. 3 Histological sections of kidney obtained from (**a**) LEW rats with isograft; (**b**) Untreated LEW rats with rejected BN renal graft; (**c**) tacrolimus (3.2 mg/kg/day) treated LEW rats with BN renal graft at more than 100 days after transplantation; (**d**) CyA (10 mg/kg/day) treated LEW rats with BN renal graft at more than 100 days after transplantation and (**e**) CyA (32 mg/kg/day) treated LEW rats with BN renal graft after grafting

treated from day 4–16 after grafting survived for more than 50 days. However, the MSTs were 19 days for recipient rats treated with CyA at 10 mg/kg per day from day 2–14, and 13 days for those treated from day 4–16 after grafting.

Discussion

The data presented here demonstrate the effect of tacrolimus on the survival of renal allografts in the rat. Tacrolimus, as primary therapy, significantly prolonged rat renal allograft in a dose-dependent manner, with the BN- to LEW strain combination. This strain combination is considered to have a strong histocompatibility difference as well as ABO incompatability [13]. The minimum effective dose was around 1 mg/kg per day, and a dose of 3.2 mg/kg per day could further prolong the survival of 5 out of 7 recipient rats indefinitely. This effect of tacrolimus on rat renal allograft survival is con-

sistent with its effect on the survival of other rat organ allografts, such as heart and liver, when administered orally [9, 20]. These results strongly suggest that the effective dose of oral tacrolimus for organ allograft survival in the rat system is at least 1 mg/kg per day. CyA, however, also significantly induced rat renal allograft survival in a dose-dependent manner in the present study, but the dose needed for such an effect is three times higher than that required with tacrolimus.

Regarding the blood trough levels in recipient rats, we found that effectivity started from 1 ng/ml in tacrolimus- and 200 ng/ml in CyA-treated groups. Interestingly, the effective blood trough level of tacrolimus was 10-fold lower in rat than that in human organ transplantation, while CyA showed almost no difference between human and rat systems [11]. As only a few studies have been done on tacrolimus blood trough levels in experimental organ transplantation in the rat system, the reasons for these results remain unclear. However, our data suggest that tacrolimus might be more effectively administered to the rat in another way (e.g., intramuscularly and subcutaneously) rather than orally.

The data obtained in this study show that there was no significant difference in renal function between rats treated with the most effective dose of tacrolimus and those treated with CyA, according to the kinetic studies of plasma creatinine. Side effects of agents in recipient rats medicated with tacrolimus and CyA after transplantation were also studied. Anorexia and emaciation were only found in the group treated with high doses of CyA (32 mg/kg per day). Body weight of both the tacrolimus- and the CyA-treated group recovered within a few days after the operation. Histological studies revealed no evidence of nephrotoxicity in either the tacrolimus- or the CyA-treated groups, although minimal tubular vacuolation was encountered in the group treated with CyA at 32 mg/kg per day. These results further indicate that tacrolimus, as a primary therapy, was at least as effective in the rat as CyA, in terms of renal graft survival, renal function, and side effects. These results also correspond with those of the clinical studies in renal transplantation by the Pittsburgh group and the Japanese tacrolimus study group – which found that the effect of tacrolimus is comparable to results achieved with conventional immunosuppression, including CyA, in clinical renal transplantation [10, 34].

From the current study, it is also clear that long-term kidney survival (more than 100 days) in the rat can be induced by treating recipients with either tacrolimus or CyA for a period of 50 days after grafting. Although the mechanisms of tacrolimus – and CyA induced long-term allograft survival remain unclear, various possibilities have been postulated, such as modulation of allograft antigen expression [5, 16], depletion/inactivation of clonal active immunoresponssive cells [7, 8], permission of leukocyte migration/microchimerism in both graft and host [21, 31, 32], activation of suppressor cells or factors [12, 25, 28]. Our data indicate that the continuous presence of specific clonal deletion/inactivation in the induction and maintenance of complete or partial unresponsiveness in allograft recipients treated with tacrolimus and CyA was at least a factor in the present experiments.

Tacrolimus and CyA have, despite their structural difference highly similar biological properties. Both drugs inhibit the same early events in T lymphocyte activation, thereby preventing T helper cells from progressing from the G_0 to the G_1 phase of the cell cycle and impairing the expression of cytokines such as IL-2 though a Ca^{2+} and calmodulin dependent signal transduction pathway that is mediated by calcineurin [17, 18]. Indeed, our observation of a decrease in the percentage of recipients with detectable serum IL-2 levels after treatment with tacrolimus and CyA was consistent with this in vitro mechanism. The serum IL-2 levels were undetectable in all recipient rats treated with the most effective dose of tacrolimus or CyA, but were detectable in 45% of the allogeneic control group at days 3 and 6 after grafting. In this experiment, the serum IL-2 levels were not increased in all recipient rats of the allogenic control group. The reason for this result may be that we tested IL-2 levels systemically rather than within local tissues. The similar modes of action of tacrolimus and CyA on the cellular and molecular basis make it easy for us to explain why both drugs, when used as primary therapy, have similar effects on preventing renal allograft rejection in rats.

An important finding of our study is that the most effective dose of tacrolimus could significantly induce rat renal allograft survival by starting administration either at day 2 or at day 4 after grafting, whereas CyA had only a weak effect when administered starting at day 2, and no effect with administration starting at day 4. This is similar to results achieved using tacrolimus and CyA as anti rejection therapy in heart transplantations studied by Ochiai, et al. [23] and in rat bone marrow transplantations by Markus et al. [19]. Allograft rejection usually starts on day 3 or 4 after grafting, and it is generally difficult to control rejection-associated lymphocytes by using conventional immunosuppressive agents. Consistent with these in-vivo results, Roelen, et al. have reported that tacrolimus was able to inhibit both "naive" and "primed" human CTLs but that CyA could only suppress "naive" human CTLs in vitro [27]. Although tacrolimus and CyA have been reported to act on the same signal transduction pathway in the T lymphocyte, it seems plausible that the mechanisms by which tacrolimus and CyA exert their immunosuppressive effect include other regulatory factors that do not necessarily have to be the same for tacrolimus and CyA [26, 27]. This is a very important difference when regarding sup-

pression of allograft rejection. These findings suggest that tacrolimus might be used not only as primary prophylactic therapy but also as anti-rejection treatment in clinical renal transplantation. However, further studies regarding the cellular and molecular mechanisms of this drug should reveal whether this is indeed the case.

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