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An immunosuppressive regimen using FTY720 combined with cyclosporin in canine kidney transplantation

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Abstract FTY720 induces apoptosis, specifically in lymphocytes, and prolongs allograft survival in rats and dogs. The purpose of this study was to define an effective range of FTY720 doses that could be combined with a suboptimal dose (10 mg/kg) of cyclosporin for canine kidney allograft recipients. The combination significantly prolonged allograft survival in all groups receiving FTY720 at a dose of 0.1, 0.3, 1.0, or 3.0 mg/kg. None of the recipients died due to notable side effects of the drug. In peripheral blood, the number of lymphocytes was extremely low, whereas the percentage of granulocytes increased during FTY720 administration. No significant difference in cyclosporin trough levels was observed between the cyclosporin-alone group and the combination groups. We conclude from the present study that FTY720 has a potent effect at an extremely low dose and a wide therapeutic window when combined with cyclosporin in canine kidney transplants.

Key words FTY720, kidney transplantation, canine · Kidney transplantation, canine, FTY720 · Canine, kidney transplantation, FTY720

Introduction

FTY720 is chemically derived from ISP-I, which is purified from culture filtrates of the fungus *Isaria sinclairii* [6]. This compound has been shown to prolong cardiac and hepatic allograft survival in rats and to inhibit concanavalin A, alloantigen, and interleukin-2-dependent cell proliferation in a dose-dependent manner [16, 17]. In vitro treatment of rat splenocytes with the drug has resulted in apoptotic cell death, characterized by the absence of surface microvilli, chromatin condensation, the formation of apoptotic bodies (through electron microscopic observation), and ladder formation on nucleosome agarose gel electrophoresis [17]. FTY720-induced cell death was observed specifically in lymphocytes, but not in bone marrow-derived cells [4]. Thymocytes from MRL-lpr/lpr mice with a mutant fas gene were sensitive to FTY720, indicating that the drug-induced apoptosis was not related to fas antigens [19]. In addition, Jurkat lymphoma cells over expressed bcl-2 genes were resistant to the drug [18]. Therefore, FTY720 displays bcl-2-associated apoptosis in lymphocytes.

In heart-allografted rats treated with FTY720 at oral doses between 0.05 and 10 mg/kg for 15 days, prolonged graft survival was observed to be dose-dependent [16]. Rat liver recipients treated with FTY720 at 0.5 mg/kg for 14 days also had prolonged survival [17]. None of the recipient rats died due to severe side effects of the drug [16, 17]. FTY720 therefore appears to have a potent immunosuppressive effect without any remarkable adverse reactions.

Past studies have shown that higher dose of FTY720 is necessary to prolong graft survival in canine kidney recipients than in rodents with cardiac and hepatic allografts [15–17]. The survival times of canine allografts were markedly prolonged by a daily administration of the drug at 10 mg/kg, starting 1 day after grafting, but not at 5 mg/kg. No notable complications were observed, not even when these dogs received a high dose of FTY720. Moreover, when combined with cyclosporin

Table 1 Graft and recipient survival with 10 mg/kg cyclosporin in combination with various doses of FTY720

FTY720 Dosage	Graft (recipient) survival in days	Median	P*
A) 0 mg/kg ($n = 5$)	7 (11), 10 (11), 11 (14), 11 (20), 12 (17)	11 (14)	
B) 0.1 mg/kg ($n = 6$)	19 (20), 20 (22), 22 (25), 66 (74) 92 (95), > 100 (> 100)	44 (49.5)	< 0.01
C) 0.3 mg/kg ($n = 6$)	17 (24), 20 (22), 48 (49), 49 (51) 73 (74), > 100 (> 100)	48.5 (50)	< 0.01
D) 1.0 mg/kg ($n = 6$)	20 (22), 39 (40), 54 (57), 59 (65) 93 (97), > 100 (> 100)	56.5 (61)	< 0.01
E) 3.0 mg/kg ($n = 5$)	17 (18), 35 (38), 59 (68), 67 (71), 72 (89)	59 (68)	< 0.01

* $P < 0.01$ compared to group A (Gehan's generalized Wilcoxon test)

Table 2 Trough levels of cyclosporin and FTY720 in the canine recipients with surviving grafts. No significant difference in the cyclosporin trough level was seen with the different FTY720 doses (Student's unpaired t-test), whereas the FTY720 trough levels increased in proportion to the increase in the dose administered

	Dose (mg/kg)		Trough level at 4 weeks after grafting	
	FTY720	Cyclosporin	Cyclosporin (ng/ml)	FTY720 (ng/ml)
A	0	10	86.6 ± 48.4[a]	
B	0.1	10	25.3 ± 5.5	2.3 ± 0.2
C	0.3	10	74.3 ± 41.0	7.0 ± 2.0
D	1.0	10	78.9 ± 21.5	20.3 ± 8.8
E	3.0	10	79.0 ± 55.2	76.5 ± 26.4

at an oral dose of 10 mg/kg, the oral administration of FTY720 at 5 mg/kg resulted in a remarkable prolongation of graft survival in the dogs in a synergistic manner [17].

The present predinical study was conducted to define an effective range of FTY720 to be combined with a suboptimal dose of cyclosporin in randomized, mongrel-to-beagle kidney transplant recipients.

Materials and methods

Drugs

FTY720 was synthesized in powder form by Yoshitomi Pharmaceutical Industries (Osaka), in cooperation with the Taito Company (Tokyo). Cyclosporin, in an oil solution (but not as Neoral), was purchased from Sandoz (Basel). Both FTY720 and cyclosporin were orally administered once a day to recipient dogs according to the protocol of each experimental group. FTY720 treatment was started 1 day before transplantation and cyclosporin on the day of operation; both were continued until the day the animal died.

Kidney transplantation

Fifteen mongrel dogs were used as donors and 30 beagle dogs as recipients in this study. Two kidneys removed from a donor were grafted into two different recipients following a standard method, with the recipients receiving bilateral nephrectomy immediately after kidney transplantation was completed. The operation was performed on all animals under general anesthesia with pentobarbital sodium and controlled respiration with a respirator (Harvard apparatus, model 55-0715). Lactated Ringer's solution was given during the operation, and furosemide was administered intravenously upon completion of the vascular anastomoses. The day of kidney transplantation was regarded as day 0 after grafting. Antibiotics were injected until 3 days after transplantation.

Graft loss was defined as either an increased level of serum creatinine higher than 0.1 mg/ml or an elevated blood urinary nitrogen level (> 2 mg/ml).

Experimental groups

Recipients were randomized by the independent study controller into five groups that were not known to the surgical team until the operation was completed. In the experimental groups, the recipients received a daily dose of 10 mg/kg cyclosporin alone or in combination with FTY720 at 0.1, 0.3, 1.0, or 3.0 mg/kg.

Laboratory examination

Peripheral blood samples were periodically obtained from the recipients. The number of white blood cells was regularly counted, as were the percentages of lymphocytes, granulocytes, and hematocrit, and the levels of serum creatinine, blood urinary nitrogen, and asparatate aminotransferase. The trough levels of cyclosporin and FTY720 in whole blood were measured by radioimmunoassay and gas chromatography-mass spectrometry at 4 weeks after grafting in the combination groups and 4 days after grafting in the cyclosporin-alone group.

Statistical analysis

Graft survival was presented as the median survival time, with comparison among groups performed by Gehan's generalized Wilcoxon test. Levels of cyclosporin and FTY720 in whole blood were expressed as mean ± standard deviation, and significant differences between the groups were assessed with Student's unpaired t-test. A probability value below 0.05 was considered significant in all studies.

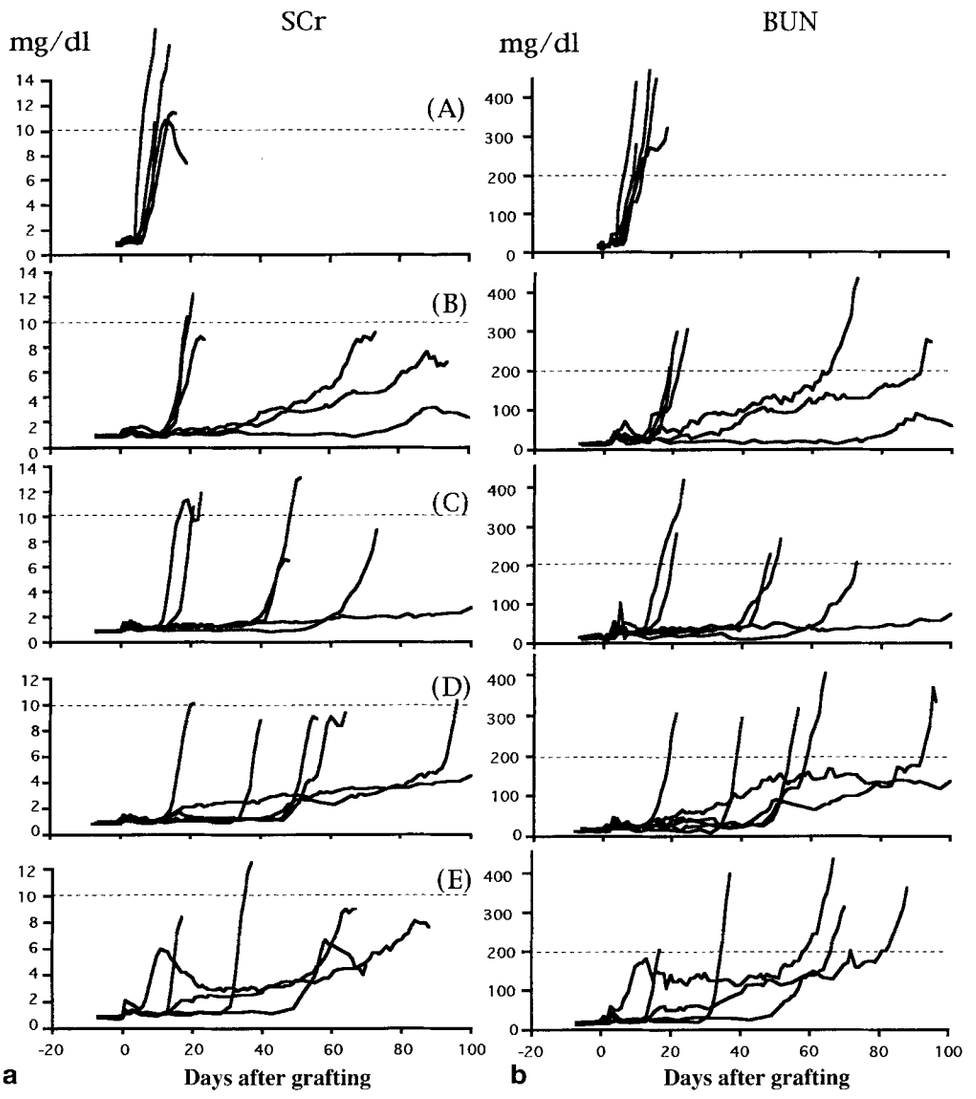

Fig. 1 a, b Time frame of **a** serum creatinine *(SCr)* and **b** blood urinary nitrogen *(BUN)* levels in recipients treated with 10 mg/kg cyclosporin combined with various doses (0.1–3.0 mg/kg) of FTY720. The combined therapy maintained low levels of SCr and BUN for at least 40 days after grafting in 14 out of 23 recipients

Ethics

All experimental protocols were conducted in accordance with the policies of the Animal Ethics Committee of the National Children's Medical Research Center.

Results

Table 1 indicates the graft and recipient survival rates in each group. Two recipients were excluded from the present study because of extremely high levels of cyclosporin (> 200 ng/ml both at 4 days and 4 weeks after grafting). None of the recipients died due to notable side effects of the drugs before graft loss. Graft survival in all combination groups (groups B to E) was prolonged significantly in comparison with the cyclosporin-alone group (group A). The survival time tended to increase with an increase in the FTY720 dosage, although there was no significant difference among the combination groups. All grafts had histologically confirmed acute rejection on the day of recipient death.

The trough levels of cyclosporin and FTY720 in recipients with surviving grafts are shown in Table 2. No significant differences were observed in the cyclosporin levels between group A at 4 days after grafting and groups B, C, D, or E at 4 weeks after grafting. In addition, the blood levels of FTY720 increased in proportion to the increase in the FTY720 dose administered.

Figure 1 shows that the levels of serum creatinine and blood urinary nitrogen in group A were markedly

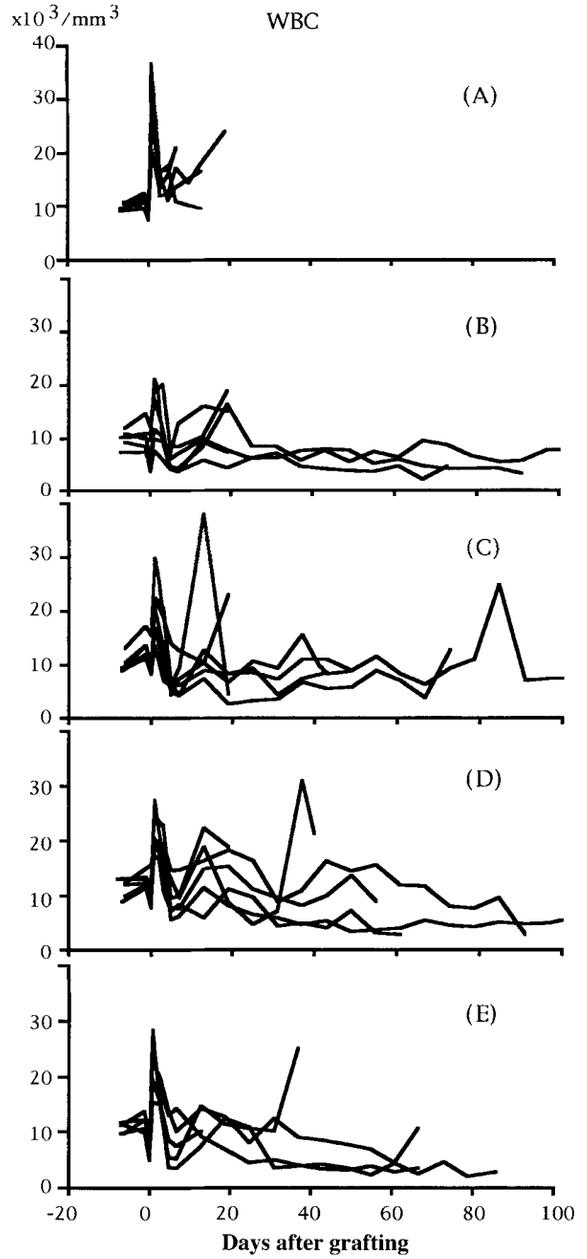

Fig. 2 A–E Time frame of white blood cell *(WBc)* count in recipients treated with cyclosporin alone and cyclosporin combined with FTY720. The number of WBC temporarily increased immediately after kidney transplantation in all recipients. Thereafter, most recipients in groups B through E showed slightly decreased counts

increased by 10 days after grafting. In contrast, the combination therapy of cyclosporin and FTY720 (groups B to E) maintained low levels of serum creatinine and blood urinary nitrogen for at least 40 days after grafting in 14 out of 23 recipients.

The white blood cell counts in the peripheral blood, shown in Fig. 2, temporarily increased immediately after kidney grafting in all of the experimental groups (groups A to E); thereafter, the counts decreased slightly in the combination groups (groups B to E). The proportion of lymphocytes was extremely low during the observation period in the groups receiving combined cyclosporin/FTY720 therapy (Fig. 3 a). The decrease in lymphocytes was accompanied by a marked increase in the percentage of granulocytes (Fig. 3 b). The hematocrit ratio decreased slightly during the early phase after grafting but tended to recover in long-term survivors (Fig. 4).

The level of serum asparatate aminotransferase temporarily increased immediately after grafting, possibly due to ischemia-reperfusion injury of the allografted kidneys. Thereafter, asparatate aminotransferase remained at low levels until the day of graft rejection, when it may have been released from the rejecting kidneys (Fig. 5).

Discussion

Currently used immunosuppressive drugs are mainly classified as either drugs which inhibit of T-cell function (cyclosporin, tacrolimus, rapamycin) or those which inhibit nucleosome biosynthesis (azathioprine, RS-61443, mizoribine). FTY720 induces bcl-2-associated apoptosis in lymphocytes [18], suggesting that the drug does not belong in either category. The drug has a potent immunosuppressive effect with an extremely wide therapeutic window in allografted rats [16]. Pretransplant administration of FTY720 has also been effective in prolonging graft survival in rat heart and liver transplants [16, 17]. This effect is completely different from that of cyclosporin or tacrolimus; pretransplant treatment of recipients with cyclosporin or tacrolimus does not prolong graft survival [8, 20].

In the present study, all grafts treated with cyclosporin (10 mg/kg, orally) alone were rejected by 12 days after grafting. This is not significantly different from the untreated control allografts that we reported on previously [17], indicating that cyclosporin at an oral dose of 10 mg/kg is not effective for canine recipients. The cyclosporin dose necessary for long-term prolongation of graft survival after canine transplantation has been reported to be 20 mg/kg or more [3, 7, 9, 14]. A significant prolongation of graft survival was observed in recipients who received 10 mg/kg cyclosporin in combination with a low dose of FTY720 (0.1–3.0 mg/kg). The trough level of cyclosporin in recipient blood was not affected by the FTY720 dose. The blood level of FTY720 increased in proportion to the dose administered. We have previously demonstrated that 5 mg/kg FTY720 alone is not an effective dose in canine kidney recipients, where the average trough level of the drug is 180 ng/ml in whole blood

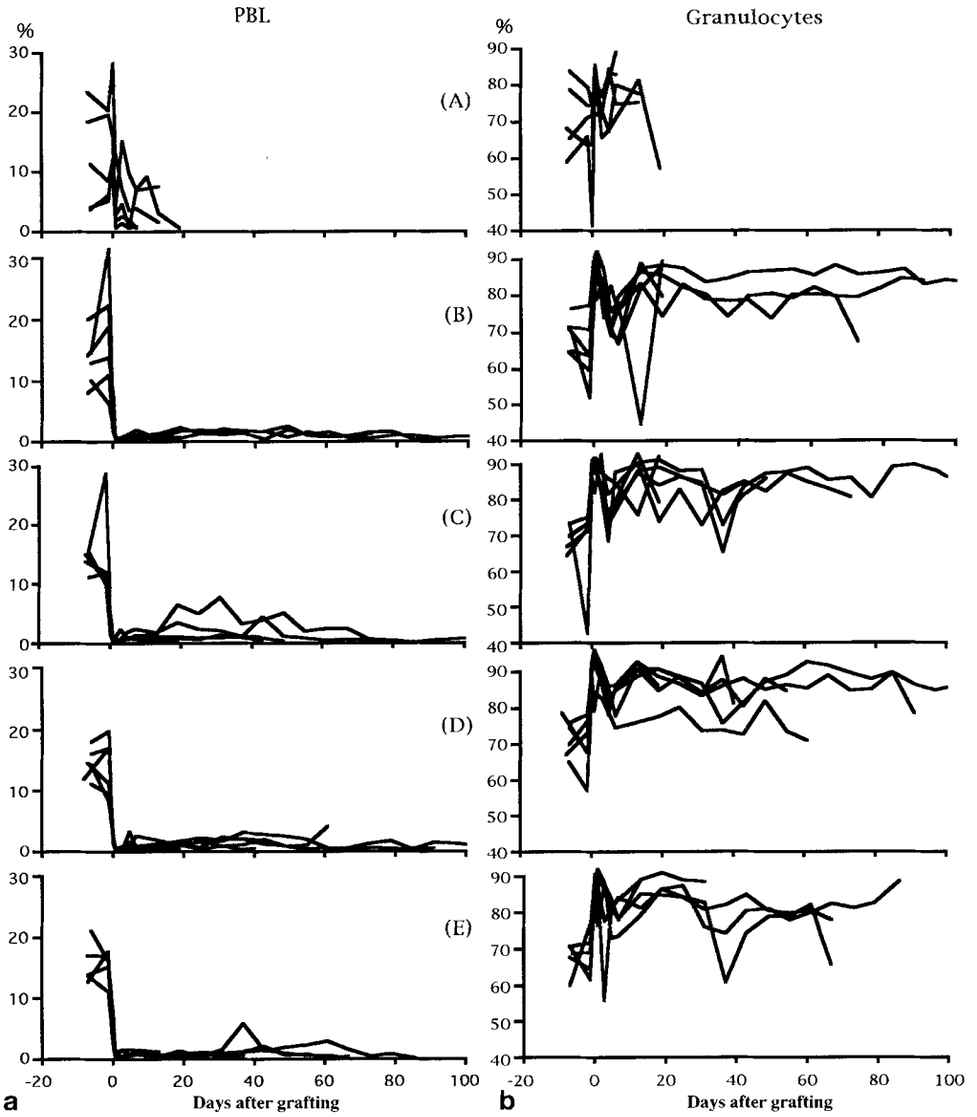

Fig. 3a,b The ratio of peripheral lymphocytes *(PBL)* and granulocytes after kidney transplantation: **a** The lymphocyte ratio was extremely low in all recipients given FTY720 during the experimental period; **b** The percentage of granulocytes increased remarkably in most recipients given FTY720

[17]. All recipients in this study had blood levels below 180 ng/ml. None of the recipients died before the onset of graft rejection. Thus, when combined with an ineffective dose of cyclosporin (10 mg/kg), FTY720 in a range of 0.1–3.0 mg/kg per day was extremely effective for long-term graft acceptance without any severe side effects. Furthermore, it has been shown that canine recipients can tolerate 10 mg/kg of FTY720 without any complications [15]. These results demonstrate that FTY720 has a wide therapeutic window, even in canine recipients. We therefore feel that the drug can be safely administered without any monitoring of blood levels.

In the groups treated with cyclosporin and FTY720 in combination, the white blood cell counts decreased slightly in the peripheral blood because of the marked reduction in the lymphocyte population, and the reduced lymphocytes resulted in an increased proportion of granulocytes. A temporary decrease in hematocrit was observed in some recipients, but this was not remarkable. Based on these data, FTY720 may be said to specifically affect canine lymphocytes as it does rat lymphocytes [4, 17]. The drug did not affect liver function.

Combined therapy with cyclosporin, azathioprine and steroids is commonly used in clinical transplantation. Long-term administration of azathioprine and steroids is known to be associated with a number of serious side effects including bone marrow dysfunction, liver function disorder, diabetes mellitus, osteoporosis, cataracts, and obesity. To minimize the complications of these drugs, immunosuppressive regimens combining

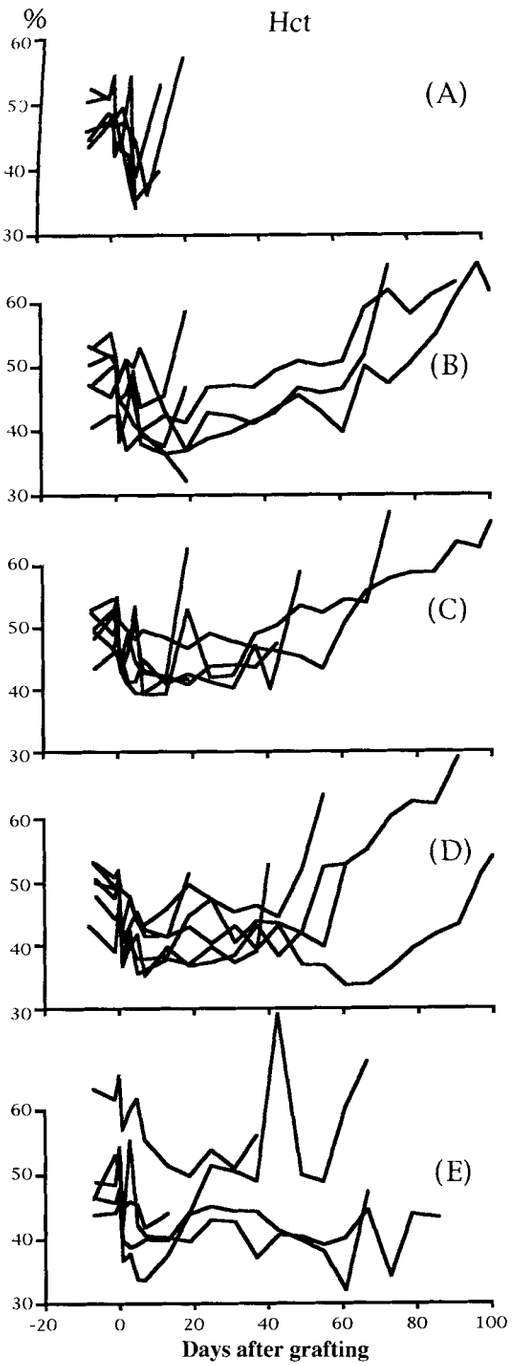

Fig. 4 A–E Time frame of the hematocrit *(Hct)* ratio in peripheral blood after kidney transplantation. The proportion of Hct in the peripheral blood decreased slightly during the early phase after grafting in the recipients with combined therapy. However, it tended to recover in the long-term survivors

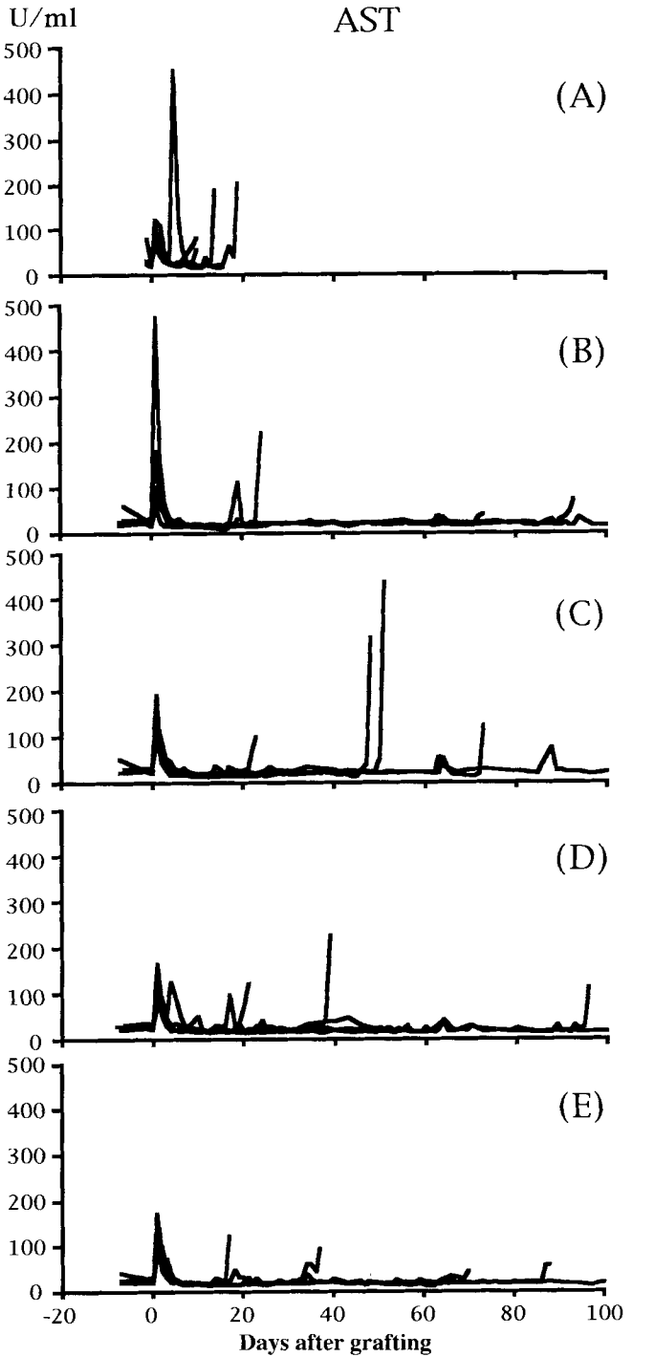

Fig. 5 A–E Levels of asparatate aminotransferase *(AST)* in the recipients given cyclosporin alone or in combination with FTY720. The level temporarily increased immediately after grafting and then remained at a low level until the day of graft rejection

cyclosporin with other drugs, such as mizoribine or RS-61443, have been used both in canine kidney transplantation [2, 10] and in clinical transplantation [5, 12, 13]. The mechanisms of action of mizoribine are fundamentally identical to those of RS-61443; both drugs selectively inhibit the de novo pathway of guanine nucleotide synthesis, resulting in lymphocyte-specific inhibition [1, 11]. However, it is still difficult to completely withhold steroids from clinical transplant recipients. The present combination therapy, using cyclosporin with FTY720, suggests the possibility of achieving steroid-free immunosuppression.

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