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COMBINED IMMUNOSUPPRESSIVE THERAPY WITH LOW DOSE FK506 AND ANTIMETABOLITES IN RAT ALLOGENEIC HEART TRANSPLANTATION

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Following rat heterotopic heart allotransplantation, low to lethal doses of the antimetabolites mizoribine (MIZ), RS-61443 (RS), and AZA were given alone or in combination with subtherapeutic doses of FK506 (0.04 mg/kg/day) for 14 days after transplantation. With the median effect analysis of Chou and Kahan for quantitative drug interactions, substantial therapeutic synergism was demonstrated between FK506 and nontoxic doses of MIZ (2.5, 5, and 10 mg/kg/day) or AZA (5, 30, and 45 mg/kg/day), which was particularly evident with the lowest dose MIZ (2.5 mg/kg/day). When FK506 was used in combination with MIZ or AZA but not with RS, the maximum effect (peak median graft survival) was enhanced significantly from 15 days (MIZ alone) to 26 days ($P<0.05$), and from 19 days (AZA alone) to 32 days ($P<0.01$). In contrast, RS interacted with FK506 no more than additively. Although RS was the most powerful single antimetabolite, the best overall survival was obtained by combining AZA and FK506. The addition of FK506 did not significantly increase the percent mortality and LD_{50} of the antimetabolites.

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The strategy of multipharmaceutical immunosuppressive therapy for organ transplantation is to enhance therapeutic efficacy while reducing the toxicity of individual drugs in the regimen. The dose-dependent side effects of FK506, including nephrotoxicity, have prompted its clinical use with other

agents that have different sites of cellular and molecular action. The antimetabolite drugs RS-61443 (RS)* (*1*), mizoribine (MIZ) (*2*), and AZA are candidates for combination with FK506 based on in vitro observations (*3*, *4*). We report here companion in vivo studies with a heterotopic heart transplantation model, in which subtherapeutic doses of FK506 were combined with low to lethal doses of MIZ, AZA, or RS. Graft survival and lethal toxicity were the end points. Using the Chou median effect analysis (*5*, *6*) advocated by Kahan (*7*, *8*) to quantitate the combined drugs, MIZ and AZA at several different doses, but not RS 61443, were synergistic with low dose FK506. Lethal toxicity of the antimetabolite drugs was not changed significantly by their use with low dose FK506.

MATERIALS AND METHODS

Animals. Inbred male rats weighing 200–300 g were purchased from Harlan Spraque-Dawley Inc. (Indianapolis, IN). Lewis rats (LEW, $RT1^l$) and ACI rats ($RT1^a$) served as recipients and donors, respectively. Animals were maintained in conventional animal facilities with water and commercial rat chow provided ad libitum.

Heterotopic heart transplantation. The operation of Ono and Lindsay (*9*) was used with anastomosis of the donor to recipient aorta and the donor pulmonary artery to the recipient inferior vena cava. Graft pulsation, body weight, activity, and defecation patterns were monitored daily after transplantation. Rejection was established by cessation of heartbeat upon abdominal palpation and confirmed by direct inspection at laparotomy and histologic examination.

Immunosuppression. All immunosuppressive drugs were continued for 14 days, starting on the day of heart transplantation. FK506 (a gift of the Fujisawa Pharmaceutical Co., Osaka, Japan) was suspended in normal saline and given intramuscularly in daily doses of 0.04 mg/kg. To this dose schedule, which was shown previously to be minimally immunosuppressive (*10*), the individual antimetabolite drugs were added.

The antimetabolite drugs were prepared daily and administered alone or added to baseline FK506 by gastric instillation in the minimal to lethal doses summarized in Tables 2, 3, and 4. RS (donated by Syntex Inc., Palo Alto, CA) was suspended in a special vehicle that contained 0.5% carboxymethylcellulose, 0.4% polysorbate 80, 0.9% benzyl alcohol, and 0.9% sodium chloride in distilled water. MIZ (Bredinin, donated by Asahi Chemical Industry Co. Ltd., Tokyo, Japan) was dissolved in distilled water. AZA (Imuran, Burroughs Welcome Co., Research Triangle Park, NC) was bought from commercial pharmacies and dissolved in distilled water.

Statistical analysis. Graft survival (in days) was calculated from the date of allotransplantation until the date of graft failure or animal death. Grafts surviving more than 100 days were right censored. The Mann-Whitney *U* test (Wilcoxon rank sum test) was used for uncensored observations to assess the effect of combined therapy on graft survival compared with each drug treatment (excluding doses with $>LD_{50}$). The maximum effect (peak median survival) of each antimetabolite alone or with FK506 was compared by using the Kruskall-Wallis test, a nonparametric test equivalent to the one-way analysis of variance, followed by the Mann-Whitney *U* test. Logistic regression was used to assess the effect of FK506 on mortality and to estimate the LD_{50}. The results were considered significant if the *P*-value was < 0.05.

The interaction between FK506 and antimetabolites was assessed using the median-effect analysis of Chou (*5*). The application of this method for in vitro and in vivo studies to quantify drug synergism or antagonism has been described previously (*5–8*). Synergism or antagonism was expressed as the combination index (CI), derived from the equation (*7*, *8*):

$$CI_X = (D_1 \text{ combined}/D_1 \text{ alone}) + (D_2 \text{ combined}/D_2 \text{ alone}),$$

where D_1 combined and D_2 combined represent the amount of drug 1 or 2, respectively, that is necessary in the combination to produce effect x. D_1 alone and D_2 alone represent the amount of drug necessary to produce the same effect x when used alone. CI values less than 1.0 suggest synergism, whereas those above 1.0 indicate antagonism, and those equal to 1.0 indicate additivity. Only therapeutic doses of antimetabolites with $<LD_{50}$ were used in the median-effect analyses.

* Abbreviations: CI, combination index; MIZ, mizoribine; RS, RS-61443.

RESULTS

Baseline FK506. As reported previously by Murase et al. (*10*) and confirmed with fresh controls, the median graft survival with baseline FK506 of 0.04 mg/kg/day was increased from 6 to 9 days (Table 1). In the earlier studies, 34-fold greater doses than this did not cause lethal toxicity, while therapeutic efficacy increased throughout (Fig. 1).

Antimetabolite drug monotherapy. Median graft survival for each of the antimetabolite agents is given in Table 2 (MIZ), Table 3 (AZA), and Table 4 (RS), and summarized from all experiments in Figure 2. With antimetabolite monotherapy, graft survival was prolonged with dose increments until this improvement was interrupted at higher doses by the mortality of overdosage. The peak median graft survival was 15 days for MIZ (at 15 mg/kg/day), 19 days for AZA (45 mg/kg/day), and 23 days for RS (40 mg/kg/day). The difference was significant between RS and MIZ ($P=0.04$) but not between RS and AZA. Indefinite graft survival (>100 days) in 2 experiments was achieved only with RS.

Antimetabolites combined with FK506. All antimetabolites when added to low dose FK506 provided better results at

TABLE 1. Graft survivals with FK506 treatment and untreated control

FK506 (mg/kg)	Graft survival days	Median
0	6,6,6,6,7,7,7	6
0.04	7,7,7,8,8,9,9,10,12,12,37	9

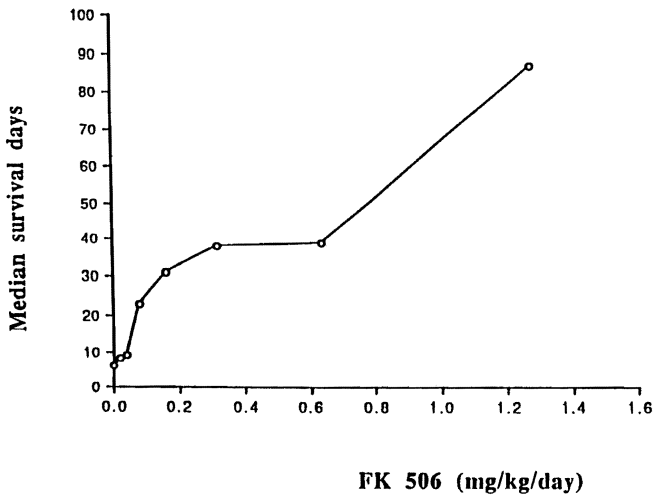

FIGURE 1. Relation of FK506 monotherapy dose (intramuscularly for 14 days) and graft survival (data for 0.08 to 1.28 mg/kg/day from Ref. *10*).

TABLE 2. The effect of MIZ treatment with or without the addition of FK506

MIZ (mg/kg/day)	FK506 (mg/kg/day)	Graft survival days[a]	Median[b]	% Mortality	LD_{50} (mg/kg/day)
2.5	0	6,7,7,7,8	7	0	
5	0	8,8,9,11,11,13	9	0	
10	0	9,9,12,14,14,15	12	0	20
15	0	11,14,15,15,16	15	0	
20	0	4*,12*,14*,20,21	14	60	
30	0	2*,5*,8*,11*,13*	8	100	
2.5	0.04	12,12,16,21,24,27,28	21	0	
5	0.04	14,16,19,20,20,26	19	0	
10	0.04	13,17,26,28,29	26	0	16
15	0.04	11*,12*,21,21,21	21	40	
20	0.04	2*,4*,12*,9*,22	9	80	
30	0.04	4*,4*,4*,4*,5*	4	100	

[a] Numbers with asterisk indicate that the rat died with a living graft.
[b] Underlined numbers indicate the maximum median graft survival obtained using MIZ alone or in combination with FK506.

TABLE 3. The effect of AZA treatment with or without the addition of FK506

AZA (mg/kg/day)	FK506 (mg/kg/day)	Graft survival days[a]	Median[b]	% Mortality	LD_{50} (mg/kg/day)
5	0	6,6,6,7,10	6	0	
30	0	7,8,11,12,17	11	0	
45	0	16,17,19,22,28	19	0	53
60	0	10*,11*,11*,13*,13*	11	100	
90	0	5*,5*,11*,21	5	75	
5	0.04	7,10,11,11,18,20	11	0	
30	0.04	26,27,28,29,29	28	0	
45	0.04	28,30,32,32,34	32[c]	0	64
60	0.04	12*,14*,30,32,32	30	40	
90	0.04	6*,6*,6*,6*,6*	6	100	

[a] Numbers with asterisk indicate that the rat died with a living graft.
[b] Underlined numbers indicate the maximum median graft survival obtained using AZA alone or in combination with FK506.
[c] Maximum survival was significantly different from those of MIZ ($P=0.02$) or RS ($P=0.04$) treatment in combination with FK506.

some doses ($P<0.05$) than when low dose FK506 was given alone. This was accomplished at doses of 2.5, 5.0, or 10.0 mg/kg/day MIZ (Table 2), 30 or 45 mg/kg/day AZA (Table 3), and only 20 mg/kg/day RS (Table 4). MIZ at a dose of 2.5 mg/kg/day had almost no effect on graft survival as a single agent, but markedly prolonged graft survival in combination with FK506. The maximum peak median graft survival was 32 days using 45 mg/kg/day AZA in combination with FK506. This was longer than with the best monotherapy doses of 10 mg/kg/day MIZ ($P=0.03$) and 40 mg/kg/day RS ($P=0.43$) (see also Fig. 2).

Combination indices with antimetabolites and FK506. The CI for MIZ calculated from foregoing data (excluding doses with $>LD_{50}$) were 0.64, 0.86, and 0.70 at doses of 2.5, 5.0, and 10.0 mg/kg/day, indicating therapeutic synergism between MIZ and FK506 (Fig. 2a). Synergism was also seen (Fig. 2b) with AZA doses of 30 or 45 mg/kg/day (CI = 0.75 and 0.80, respectively). However, a CI of 2.27 at the AZA dose of 5 mg/kg/day suggested antagonism at this lower dose. The interaction between RS and FK506 was additive (CI = 1.00), but only at the RS dose of 20 mg/kg/day (Fig. 2c). The slight enhancement of the maximum effect observed at 40 mg/kg/day was classified as "antagonism" (CI = 1.12).

Toxicity of antimetabolites with and without FK506. The toxic dose with antimetabolite monotherapy was ≥20 mg/kg/day for MIZ (Table 2) and ≥60 mg/kg/day for AZA (Table 3) or RS (Table 4). Percent mortality at each dose of antimetabolite was not affected significantly by combination with FK506. Animals with toxic doses of antimetabolites developed diarrhea and continuous weight loss, and some died with functioning grafts. At the time of necropsy, severe edema of the intestinal mucosa was the most common finding. Lethal dose MIZ treatment frequently induced scattered hemorrhage and ulceration of the intestinal mucosa, which sometimes led to perforation.

The LD_{50} for each antimetabolite was calculated using linear logistic regression. These were 20 mg/kg/day for MIZ alone versus 16 mg/kg/day for MIZ+FK506; 53 mg/kg/day for AZA alone versus 64 mg/kg/day for AZA+FK506; and 65 mg/kg/day for RS alone versus 55 mg/kg/day for RS+FK506. The minor increases or decreases of LD_{50} doses with addition of FK506 to the individual metabolites (Tables 2–4) were not significant.

DISCUSSION

In analyzing our results, we accepted Berenbaum's (*11*) rigorous definition of "synergism" based on the dose-response relationship and have quantitated synergism by the median effect analysis of Chou (*5*) and Chou and Talalay (*6*), which has been advocated by Vathsala et al. (*7*) and Kahan et al. (*8*) for in vivo as well as in vitro models. This method quantifies the interactions between 2 immunosuppressive agents at each dose combination as expressed by the CI. In our study, we calculated the CI at several dose combinations of the antimetabolite agents added to a fixed low dose of FK506 and

TABLE 4. The effect of RS treatment with or without the addition of FK506

RS (mg/kg/day)	FK506 (mg/kg/day)	Graft survival days[a]	Median[b]	% Mortality	LD_{50} (mg/kg/day)
20	0	8,10,11,13,17,18,21	13	0	
40	0	13,18,23,25,26,30	23[c]	0	65
60	0	5*,13*,19,20,>100,>100	19	33	
80	0	3*,4*,4*,4*	4	100	
20	0.04	19,20,22,23,24,25	22	0	
40	0.04	24,25,27,31,34,	27	0	55
60	0.04	12*,13*,13*,33,>100	13	60	
80	0.04	4*,4*,5*,5*	4	100	

[a] Numbers with asterisk indicate that the rat died with a living graft.
[b] Underlined numbers indicate the maximum median graft survival obtained using RS alone or in combination with FK506.
[c] Maximum survival was longer than that of MIZ monotherapy ($P<0.05$) and RS monotherapy (NS).

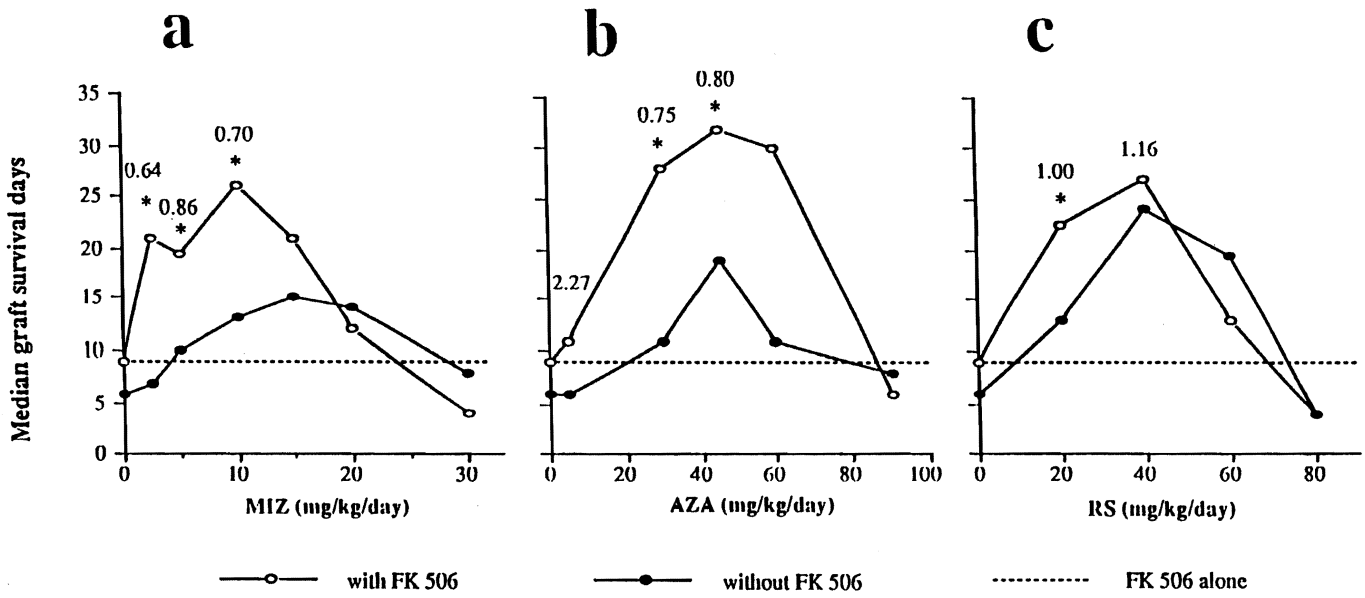

FIGURE 2. Immunosuppressive effect on ACI-LEW heterotopic heart graft survival of various antimetabolite drugs given for 14 days alone (lower curves) or added to 0.04 mg/kg/day FK506 (upper curves). The combination index (CI numbers within the figure) was calculated from the graft survival achieved with each antimetabolite treatment at doses $<LD_{50}$, and used to quantitate drug synergism between different doses of the antimetabolites and a fixed low dose of FK506. *Survival significantly greater ($P<0.05$) than with antimetabolite monotherapy and fixed low dose FK506 monotherapy.

also compared the maximum effect of each of these drugs when given alone.

A benefit of combined immunosuppressive therapy for heart transplantation from ACI to LEW rats was clearly shown. Therapeutic synergism was present when FK506 was combined with either MIZ or AZA, whereas RS interacted with FK506 no more than additively. Therapeutic synergism was pronounced at the lowest dose of MIZ (2.5 mg/kg/day), but not with low dose AZA, which was antagonistic (5 mg/kg/day). Low dose RS was not tested with FK506. Of the 3 antimetabolites, RS gave the best graft survival when used alone, but the best results were obtained by combining AZA and FK506.

A conventional explanation for drug synergism is the different site of action of the combined agents. Suppression of allospecific T cell immunity by FK506 is explained by inhibition of the production of IL-2 and its receptor (*12*). In contrast, MIZ, RS, and AZA inhibit purine biosynthesis, curtailing the DNA synthesis necessary for cell division, including that of activated T and B lymphocytes (*1, 2, 13*). Thus, antimetabolites act at a late stage of the cell activation pathway by blocking the cell cycle between G_1 to S (*14*), whereas FK506 acts at an early stage of T cell activation at the G_0 to G_1 transition. Synergism resulting from these diverse antilymphocytic mechanisms has been observed in in vitro test models, but less predictably so in vivo. In the whole animal, confounding factors that could cause divergence of in vitro from predicted in vivo results may include the alteration by each drug of the other agent's metabolism and elimination. In addition, an increasingly obfuscating recognized factor is a rapidly evolving change in host immune reactivity caused by ubiquitous engagement of migratory donor leukocytes that graft and mingle with immune cells of the recipient and survive for the life of the graft (*15*). Thus, the need for testing in whole animals versus reliance on surrogate in vitro models is evident.

In addition to the desired effect of any drug combination, its utility depends on toxic interactions. In our experiments, the mortality of the recipients and the calculated LD_{50} did not change significantly by adding low dose FK506 to any of the

tested antimetabolites. Positive enhancement of the therapeutic effect without increasing toxicity indicates an improvement in therapeutic index ($TI=LD_{50}:ED_{50}$ ratio) for these agents, with the promise of an increased clinical benefit. However, clinically relevant side effects in the therapeutic range could not be decisively assessed in this study. The rat model has little value for evaluating the adverse effects of FK506 in humans which have been broadly classified under nephrotoxicity, alteration in carbohydrate metabolism, neurotoxicity, and susceptibility to infection and malignancy (*16*).

The introduction of CsA and, more recently, FK506 has dramatically reduced the incidence and severity of acute cellular rejection, but the risk of chronic rejection associated with proliferative arteriopathy remains high (*17–20*). Although the pathogenesis of these vascular changes is disputed, humoral antibodies are suspected to play a key role. Previous reports have suggested the utility of AZA and RS in reducing chronic vascular rejection (*21*, *22*). Because these drugs inhibit both T and B cell activity, combined immunosuppressive therapy that includes antimetabolites may offer a more effective means of preventing humoral as well as cellular rejection. Recently, the combination of FK506 with a wide range of antimetabolites has been shown to mitigate the humoral rejection of hamster xenografts in rats to an extent far greater than with FK506 alone (*23*).

In conclusion, we have demonstrated that after rat heterotopic heart allotransplantation there is therapeutic synergism between MIZ or AZA, but not RS, and a fixed low dose of FK506. The lethal toxicity of the antimetabolites alone was not affected by the addition of the FK506. The effect of higher dose FK506 in such combination therapy also remains to be elucidated.

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