Immunosuppressive Effect of FK-506 on In Vitro Lymphocyte Alloactivation: Synergism With Cyclosporine A

A. Zeevi, R. Duquesnoy, G. Eiras, H. Rabinowich, S. Todo, L. Makowka, and T.E. Starzl

RECENT STUDIES have described a new potent immunosuppressive drug FK-506 (FK) that induces considerable prolongation of allograft survival.[1-3] This drug is isolated from *Streptomyces tsukubaensis*, has a molecular weight of 822 daltons, and is structurally different from cyclosporine A (CsA). In vitro investigations have demonstrated a strong inhibitory effect on lymphocyte activation that appears to be mediated by blocking of interleukin 2 (IL-2) release.[4] Our studies have extended these findings by showing that FK inhibits secondary proliferation of alloreactive T lymphocytes generated from mixed lymphocyte reaction (MLR) cultures or propagated from organ transplant biopsy specimens with cellular infiltrates.[5] The in vitro immunosuppressive effect of FK is generally several hundredfold greater than that of CsA. On the other hand, FK does not inhibit secondary proliferation of activated T cells stimulated by IL-2.

With respect to the possibility that FK could be used together with CsA as an immunosuppressive drug regimen to prolong allograft survival, we studied in vitro effects of low doses of these drug combinations on lymphocyte proliferation. The results demonstrate synergism between FK and CsA in their inhibition of both primary lymphocyte alloactivation and secondary proliferation of alloreactive T cells. This in vitro synergism may have clinical application in that low doses of FK and CsA combinations may be effective in increasing transplant survival.

From the Department of Immunopathology, University of Pittsburgh.

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Address reprint requests to A. Zeevi, PhD, University of Pittsburgh, Department of Immunopathology, One Children's Place, Room 5711, Pittsburgh, PA 15213-3417.

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MATERIALS AND METHODS

MLR-Induced Proliferation

Human peripheral blood lymphocytes were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. Undirectional MLR cultures were set up with 10^5 responder and 10^5 irradiated (2,000 R) stimulator cells in a volume of 200 μL of tissue culture medium (TCM) supplemented with 10% human serum and incubated for six days. During the final 20 hours of incubation, each culture was labeled with 1 μCi of ^{3}H-thymidine. The cultures were harvested and counted in a liquid scintillation counter.

Primed Lymphocyte Test

Alloreactive lymphocyte cultures were propagated from human heart and liver transplant biopsy specimens in the presence of IL-2 as previously reported.[6,7] Secondary proliferation of alloreactive T cells was assessed in a three-day primed lymphocyte test (PLT) whereby 10^4 responder cells were incubated with 10^5 irradiated (2,000 R) stimulator cells. Lymphocyte proliferation was determined by ^{3}H-thymidine incorporation as described earlier.

Drug Sources

FK was supplied by the Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan. This drug was received as a crystalline powder, and before use it was dissolved in methanol and further diluted in TCM. CsA was obtained from Sandoz, Inc, Hanover, NJ. It was dissolved in ethanol and further diluted in TCM to various concentrations.

Dose Effects of FK and CsA on Lymphocyte Proliferation

The inhibitory effects of FK and CsA on MLR reactivity and PLT response of alloreactive lymphocytes were measured at different concentrations of drugs ranging from 0.06 to 500 ng/mL. The results were expressed as

percent inhibition by using the following formula: percent inhibition = 1 − (cpm with drugs × 100/cpm without drugs).

Pretreatment of Alloreactive T Cells With CsA and FK

Heart biopsy specimen–propagated alloreactive T cells were cultured with 20 IU/mL of recombinant IL-2 with and without 10 μg/mL of CsA or various concentrations of FK (1 μg/mL, 0.1 μg/mL, and 0.01 μg/mL). After four days of incubation, the cell cultures were washed and tested for cell viability. The cells were then tested for donor-specific PLT reactivity by incubating 10^4 cells with 10^5 irradiated stimulators for three days. Control cultures were tested for proliferation in the presence of IL-2. In addition, these pretreated cells were tested for their sensitivity to FK and CsA in PLT assays.

RESULTS

Synergism Between FK and CsA in Inhibition of Lymphocyte Activation

Studies on lymphocyte proliferation in primary MLR have previously shown that inhibition was observed at concentrations of FK that were several hundredfold lower than those of CsA.[4] An example of the differences between FK and CsA is shown in upper part of Fig 1. It may be seen that significant inhibition of greater than 75% was observed at concentrations of 1 ng FK/mL and 500 ng CsA/mL. The bottom part of Fig 1 illustrates that combinations of 0.1 ng/mL of FK and 10 ng/mL or 1 ng/mL of CsA induced considerable MLR inhibition, whereas at these low concentrations neither drug had any significant effect.

Another example of the synergism between low doses of FK and CsA is shown in Fig 2, which depicts the results of studies on donor-specific secondary proliferation of alloactivated T lymphocytes propagated from liver transplant biopsy material. The upper part of Fig 2 shows that the inhibition of the PLT response of these cells by FK was effective at a 400-fold lower concentration than that of CsA. The lower part of Fig 2 demonstrates that a combination of low doses of FK and CsA exerted significant inhibitory effects,

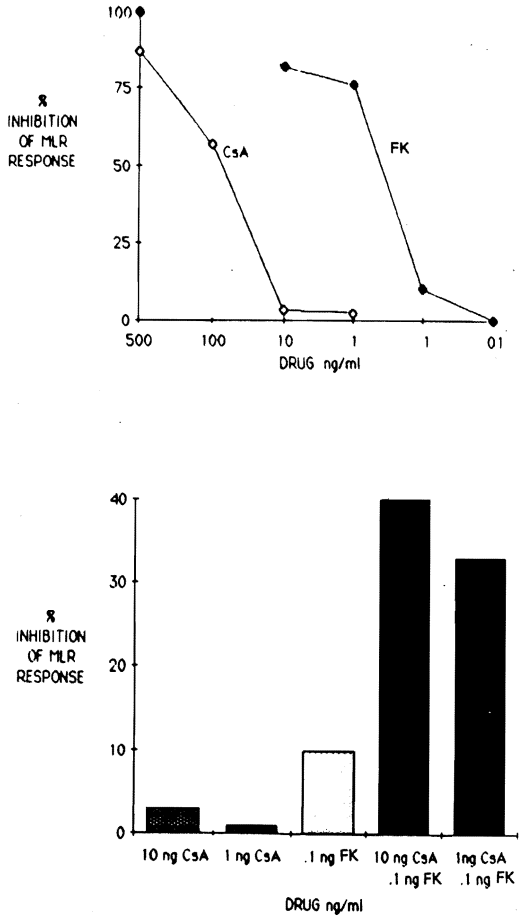

Fig 1. Synergism between FK and CsA inhibition of MLR response.

whereas at these concentrations each of these drugs alone had no significant effect.

These findings demonstrate that at very low concentrations FK and CsA have synergistic inhibitory effects on both primary and secondary T lymphocyte activation.

Effect of FK and CsA Treatment of Alloreactive T Cells

Studies were designed to determine whether pretreatment of alloactivated T cells with FK or CsA affected subsequent secondary donor-specific proliferation. Because these drugs have no effect on IL-2–induced proliferation of activated T cells (Table 1), experiments were set up whereby heart biopsy

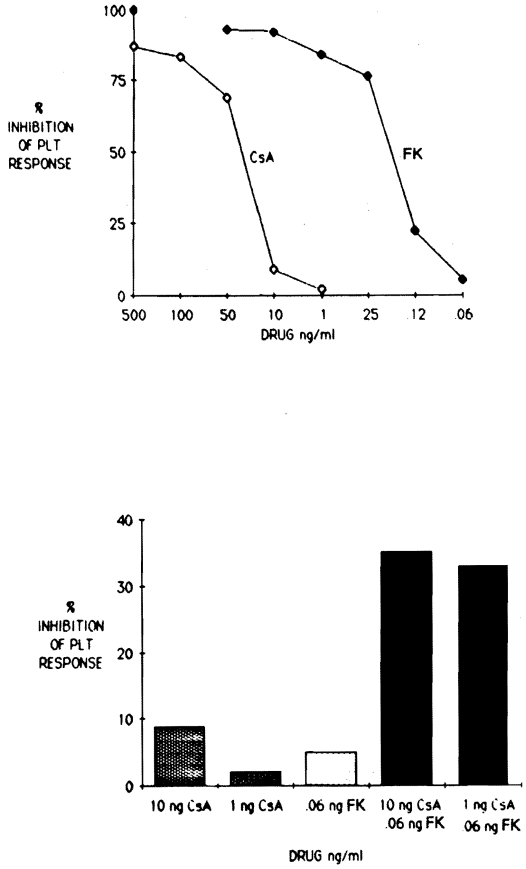

Fig 2. Synergism between FK and CsA inhibition of the PLT response of liver biopsy specimen–propagated lymphocytes.

material–grown lymphocytes were expanded with IL-2 in the presence of FK or CsA at various concentrations. After four days in culture, these expanded alloreactive T cell cultures were washed and then restimulated with donor cells in three-day PLT assays. The results shown in Table 2 demonstrate that pretreatment with FK at 1 μg/mL caused significant inhibition of PLT stimulation four days later but had no effect on IL-2–induced proliferation. On the other hand, pretreatment with CsA or lower doses of FK had no significant effect on the subsequent PLT response.

Table 1. Lack of Inhibitory Effect of FK and CsA on IL-2–Induced Proliferation of Alloreactive T Cell Clone DB29

Drug	Dose (μg/mL)	IL-2 Response (cpm)
Nothing	None	235
IL-2	None	5,296
IL-2 + FR	0.1	5.085
IL-2 + CsA	1	5,718

The MLR-generated alloreactive T cell clone DB29 is specific for a DQwl-associated cellular determinant.

Effect of FK and CsA Pretreatment on Drug Sensitivity of Alloreactive T Cells

The PLT response of pretreated cells was also tested for its susceptibility to inhibition by CsA (Fig 3). Pretreatment of IL-2–expanded heart transplant biopsy specimen–grown lymphocytes with 10 μg/mL CsA yielded cells whose PLT reactivity had essentially the same CsA sensitivity as control cells that were cultured without drugs. On the other hand, pretreatment with FK at 0.1 μg/mL yielded cell cultures whose PLT reactivity was considerably more sensitive to CsA, and strong inhibition was observed at concentrations as low as 0.05 μg/mL of CsA. It may be seen in Fig 3 that pretreatment with FK at 0.01 μg/mL also yielded cells with increased CsA sensitivity of their PLT responses.

Further studies were conducted to determine whether pretreatment with CsA or FK leads to an increased sensitivity of alloactivated T cells to FK (Table 3). Heart transplant biopsy specimen–grown lymphocytes expanded with IL-2 in the presence of 10 μg/mL CsA showed similar FK sensitivity of their donor-specific PLT response as control

Table 2. Effect of Pretreatment With FK or CsA on the IL-2 Responsiveness and Donor-Specific Reactivity of Lymphocytes Propagated From Heart Biopsy Material

Pretreatment		Proliferation Responses (cpm)		
Drug	Dose (μ/mL)	Background	IL-2	PLT
Control	None	534	22,056	66,447
FK	1	455	30,834	8,126
FK	0.1	449	29,806	81,242
FK	0.01	436	30,638	102,045
CsA	10	351	15,535	57,889

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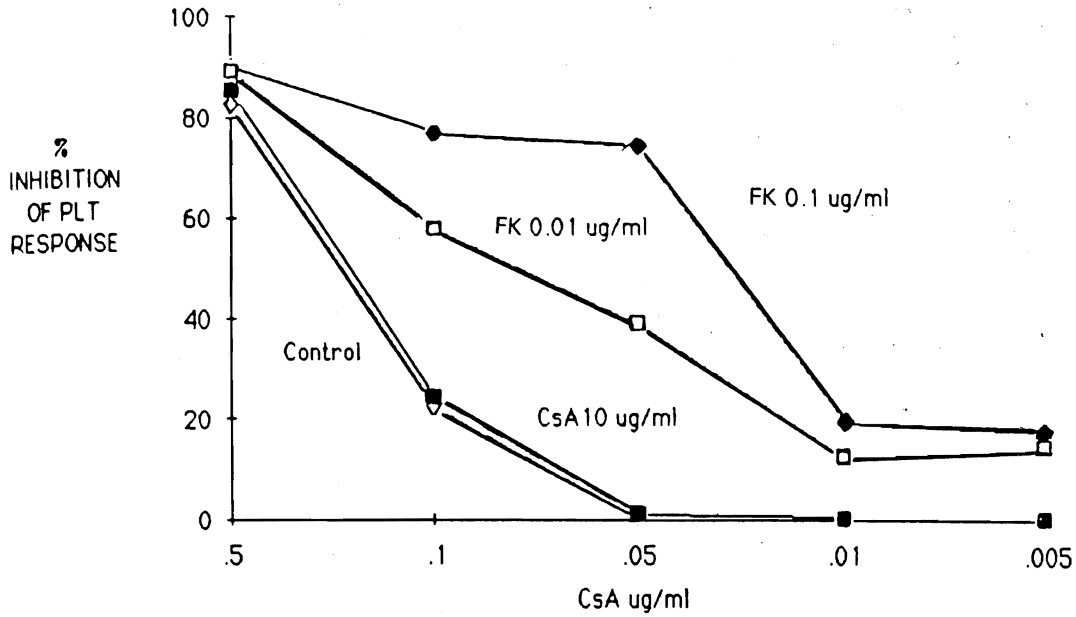

Fig 3. Increased CsA sensitivity of heart biopsy specimen–grown lymphocytes pretreated with FK.

cell cultures grown without drugs. On the other hand, pretreatment with low doses of FK yielded cells that showed increased sensitivity to FK, although the effect was not as impressive as the enhanced sensitivity to CsA shown in Fig 3.

DISCUSSION

These findings demonstrate synergism between the immune suppressive effects of low doses of FK and CsA in two types of in vitro assays. The first deals with a simultaneous incubation of these drugs during primary or secondary alloactivation of human T lymphocytes. The other assay shows synergism through sequential exposure of alloreactive lymphocytes to FK followed by CsA. The synergism between FK and CsA suggests that low-dose combinations of these drugs may be effective in prolonging allograft survival. Indeed, our experience with cardiac allografts in rats have shown a strong immunosuppressive effect of combinations of FK and CsA.[2]

An advantage of using combinations of low doses of FK and CsA to prolong transplant survival is that there would be a reduction of undesirable side effects of these drugs. For instance, the nephrotoxicity of CsA in trans-

Table 3. Inhibition of PLT Reactivity of Pretreated Heart Biopsy Specimen–Grown Lymphocyte Cultures in the Presence of Various Doses of FK

FK (μg/mL)	Percent Inhibition of PLT Response HB Cultures*			
	Control	CsA (10 μg/mL)	FK (0.1 μg/mL)	FK (0.01 μg/mL)
0.1	95	94	95	95
0.01	93	93	93	94
0.001	86	89	90	87
0.00025	0	0	53	21
0.00012	0	0	51	13

*Lymphocytes propagated from heart biopsy (HB) material were cultured with IL-2 in the presence of drugs (CsA and FK) for four days before the PLT inhibition test.

plant patients could be markedly reduced if lower doses are used in combination, perhaps with low doses of FK.

Little is understood about the mechanism of the synergism between FK and CsA. For both drugs, the inhibitory effect of lymphocyte proliferation is mediated by blockage of IL-2 release (Kino et al[4] and unpublished data). On the other hand, neither drug causes significant inhibition of IL-2–induced proliferation. The presence of IL-2 receptors on activated T cells is required for this proliferation. The appearance of IL-2 receptors on MLR-activated cells has been shown to be inhibited by FK.[4] This would mean that such cells would be less responsive to the stimulating effects of IL-2. However, our current studies do not support this notion because FK pretreatment of alloreactive T cells expanded with IL-2 did not render these cells less responsive to subsequent IL-2 stimulation, although their PLT responsiveness was impaired. Recent studies at our institution have shown that FK pretreatment of lymphocytes increases the binding of CsA.[8] These findings suggest that the synergism between FK and CsA might be related to lymphocyte membrane binding of these drugs. Studies are currently in progress to obtain a better understanding of the mechanism of this synergism.

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