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Inhibition of T-lymphocyte activation by the immunosuppressive drug FK-506

J. E. KAY, C. R. BENZIE, M. R. GOODIER, C. J. WICK & S. E. A. DOE *Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Brighton*

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SUMMARY

Nanamolar concentrations of the immunosuppressive drug FK-506 inhibit the induction of T-lymphocyte proliferation by the lectins concanavalin A (Con A) and phytohaemagglutinin (PHA). Activation by Con A is more sensitive to inhibition than the response to PHA. FK-506 inhibits an early Ca^{2+}-dependent step in the activation process, and its effects are not reversible by the addition of recombinant interleukin-2 (IL-2) or lymphokine-rich culture supernatant. While the effects of suboptimal concentrations of FK-506 and cyclosporin A (CsA) are additive, FK-506 does not enhance the effects of optimal concentrations of CsA. Both drugs also have similar effects on the expression of specific mRNA in Con A-activated lymphocytes. A brief preincubation of unstimulated cells with FK-506 irreversibly inhibits their subsequent responsiveness to Con A. The mechanism of action of FK-506 thus resembles that of CsA, except that it is effective at two to three orders of magnitude lower concentrations and its effects are much less readily reversible.

INTRODUCTION

FK-506 is a neutral macrolide of known structure isolated from the fermentation broth of a strain of *Streptomyces tsukubaensis* (Kino *et al.*, 1987a). It has a strong *in vivo* immunosuppressive action, and in experimental systems has been shown to prevent transplant rejection (Ochiai *et al.*, 1987a, b; Thomson, 1989) and the development of experimental autoimmune disease (Inamura *et al.*, 1988). Antibody synthesis is also inhibited, probably due to interference with the functioning of T-helper lymphocytes (Kino *et al.*, 1987a; Woo, Stephen & Thomson, 1988). FK-506 inhibits the proliferation *in vitro* of human and murine T lymphocytes in the mixed lymphocyte reaction, and the response to specific antigen of murine T-cell clones (Kino *et al.*, 1987a, b, c; Zeevi *et al.*, 1987; Sawada *et al.*, 1987). It also prevents the formation of IL-2 and other lymphokines, the expression of IL-2 receptors and the development of T-cytotoxic lymphocytes in such cultures.

The objective of the experiments reported here was to determine whether FK-506 also inhibited the activation of lymphocytes by lectins mitogenic for T lymphocytes, and to determine the stage of the activation process at which it exerted its effects.

MATERIALS AND METHODS

Materials

FK-506 was kindly provided by the Fujisawa Pharmaceutical Company, Osaka, Japan, and CsA by Dr J. F. Borel, Sandoz, Basle, Switzerland. FK-506 was dissolved in ethanol, and CsA in ethanol-Tween 80 as described by Wiesinger & Borel (1979). Appropriate concentrations of solvents were added to all control cultures. PHA (PHA-P from Difco Laboratories, Detroit, MI), Con A (from Flow Laboratories, Irvine, Ayrshire), human recombinant IL-2 (Cetus, Emeryville, CA), [^{35}S]L-methionine and [5-^{3}H]thymidine (1000–1350 and 5 Ci/mmol, respectively, from Amersham International, Amersham, Bucks) were dissolved or diluted in Eagle's minimal essential medium (EMEM).

Correspondence: Dr J. E. Kay, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K.

Preparation and culture of pig lymphocytes

Lymphocytes were prepared from defibrinated pig blood by a procedure described in detail elsewhere (Kay *et al.*, 1975), that includes initial sedimentation of most erythrocytes with dextran, filtration through a cotton-wool column and centrifugation on Ficoll–Hypaque step gradients. Lymphocytes were cultured at 2×10^6/ml in EMEM supplemented with 5% heat-inactivated fetal calf serum. Mitogens and inhibitors were added simultaneously unless otherwise indicated.

Lymphocyte activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein or of [^{3}H]thymidine into DNA during a terminal 4-hr pulse, as described previously (Walls & Kay, 1982). One microcurie of radioisotope was added to each of three or more replicate cultures. [^{35}S]methionine incorporation by individual cultures was almost invariably within $\pm 10\%$ of the mean for replicate cultures and usually within $\pm 5\%$. When [^{3}H]thymidine incorporation was assessed, variation was within $\pm 20\%$ of the mean values shown.

Extraction and hybridization of RNA

Lymphocytes (10^8) were lysed in Triton X-100 and cytoplasmic mRNA was extracted with phenol/chloroform as described elsewhere (Kumagai *et al.*, 1987). RNA (20 μg/lane) was then denatured, separated on a 1·1% agarose gel and transferred to a 'Gene Screen' nylon membrane. Northern blots were then hybridized to [^{32}P]-labelled DNA probes for c-myc (Dalla Favera *et al.*, 1982) and the T-cell receptor β-chain (TcRβ) (Yoshikai *et al.*, 1984) as described elsewhere (Thomas, 1980). The TcRβ probe, JUR β1, was used with kind permission of T. Mak. After hybridization, blots were washed and exposed to X-ray film (Fuji RX) for 72 hr with intensifying screens.

RESULTS

Inhibition of T-lymphocyte activation

The effects of increasing concentrations of FK-506 on the Con A- and PHA-induced activation of peripheral blood lymphocytes are shown in Fig. 1. Both the rate of incorporation of [^{35}S]methionine into protein, measured 24 hr after lectin addition, and the rate of incorporation of [^{3}H]thymidine into DNA, measured after 48 hr, were progressively inhibited by FK-506 concentrations above 0·1 nM. The concentration curves are very similar to those reported previously for the human and murine mixed lymphocyte reactions and the response of murine T-cell clones to specific antigen (Kino *et al.*, 1987a, b, c; Zeevi *et al.*, 1987; Sawada *et al.*, 1987), except that the extent of the inhibition is less complete. Maximal inhibition was seen at FK-506 concentrations of 1–10 nM, but a part of the response was resistant to inhibition even when concentrations as high as 100 nM were employed. FK-506 did not have any significant effect on the low rate of protein synthesis exhibited by unstimulated lymphocytes (4 ± 5% inhibition in 25 comparisons using 10 nM FK-506).

It is apparent from Fig. 1 that a higher proportion of the response was sensitive to inhibition by FK-506 when Con A was used as mitogen than when PHA was employed. This difference was observed in every experiment in which direct comparison was made, and whether [^{35}S]methionine or [^{3}H]thymidine incorporation was assessed. The mean percentage inhibition of the increase in the rate of protein synthesis at 24 hr was 77 ± 6% (21 experiments) when Con A was used as mitogen, compared to only 46 ± 9% (seven experiments) when PHA was employed. We have previously reported that the Con A response shows a similar greater sensitivity to inhibition by CsA than the response to PHA (Kay & Benzie, 1983).

The inhibition by FK-506 was observed at all effective mitogen concentrations (Fig. 2), though the drug was somewhat more effective at suboptimal mitogen concentrations. This was particularly the case when [^{3}H]thymidine incorporation into DNA was determined (Fig. 2b). Similar results were obtained with PHA as mitogen (data not shown).

The step in activation inhibited

When the addition of FK-506 was delayed for even a few hours after the addition of mitogen, the magnitude of the inhibition observed was greatly reduced (Fig. 3). Significant reduction in inhibition was observed in each of three similar experiments when FK-506 was added as little as 2 hr after Con A, and when the FK-506 addition was delayed for 8–12 hr its effectiveness was completely lost.

The development of such resistance to the effects of FK-506 was completely dependent on the availability of calcium ions in the lymphocyte culture medium. When Ca^{2+} were removed from the culture medium by addition of EGTA for the first 6 hr of culture, FK-506 readded at 6 hr together with Ca^{2+} was just

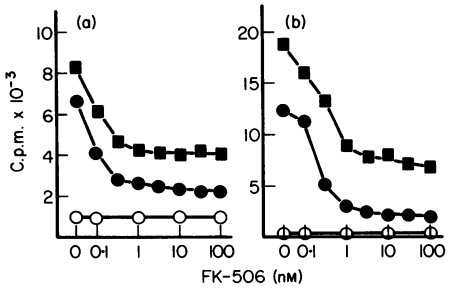

Figure 1. Effect of FK-506 concentration on the rates of incorporation of [^{35}S]methionine into protein at 24 hr (a) or of [^{3}H]thymidine into DNA at 48 hr (b) by unstimulated lymphocytes (○) or lymphocytes stimulated by Con A (●) or PHA (■).

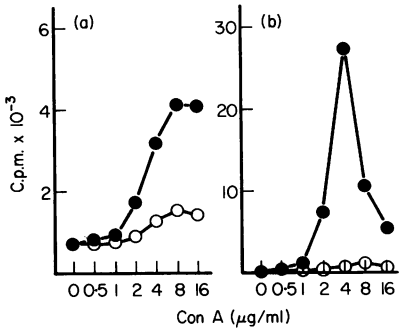

Figure 2. Effect of 10 nM FK-506 (○) added together with mitogen on the rates of incorporation of [^{35}S]methionine into protein at 24 hr (a) or of [^{3}H]thymidine into DNA at 48 hr (b) by lymphocytes incubated with the concentrations of Con A indicated.

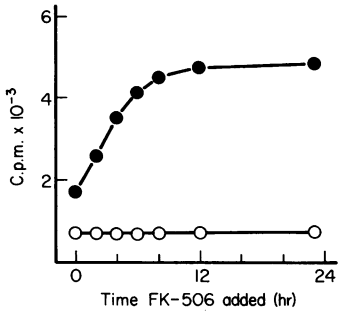

Figure 3. Effect of delayed addition of FK-506. Lymphocytes were incubated with (●) or without (○) Con A, and the rate of incorporation of [^{35}S]methionine into protein assessed at 24 hr. Con A addition was at 0 hr and 10 nM FK-506 was added either simultaneously with Con A or at the time shown thereafter.

Table 1. Dependence of the development of resistance to the effects of FK-506 on the availability of Ca^{2+}

Con A	Ca^{2+} removal	No FK-506	10 nM FK-506 at 0 hr	10 nM FK-506 at 6 hr
–	—	614±23	637±18	606±34
+	—	3510±176	1283±38	2696±78
+	0–6 hr	1952±36	890±10	881±30
+	1–6 hr	2307±88	997±46	992±39

Lymphocytes were incubated with or without Con A, and the effects of 10 nM FK-506, added at the same time as mitogen or 6 hr later, assessed by determination of [^{35}S]methionine incorporation after 24 hr. At the start of the experiment cells were washed and resuspended in Ca^{2+}-free EMEM supplemented with 5% heat-inactivated fetal calf serum. Residual Ca^{2+} were then removed by addition of 0·5 mM EGTA, and restored by addition of $CaCl_2$ to a final concentration of 2 mM. Control cultures had the same amounts of EGTA and $CaCl_2$ added simultaneously at 0 hr.

Values represent mean c.p.m. incorporated per culture ±SD.

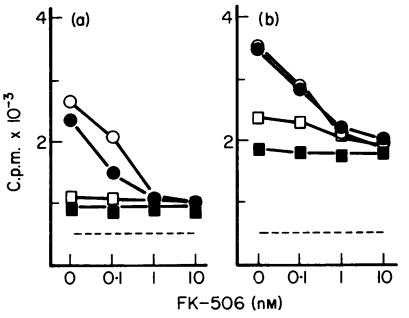

Figure 4. Interaction between FK-506 and CsA. Lymphocytes were stimulated with either Con A (a) or PHA (b), in the presence of the concentration of FK-506 shown together with 1 μg/ml CsA (■), 100 ng/ml CsA (□), 10 ng/ml CsA (●) or without CsA (○). The rate of [^{35}S]methionine incorporation into protein was determined at 24 hr. The rate of incorporation by unstimulated cultures (with or without FK-506) is indicated by the dashed line.

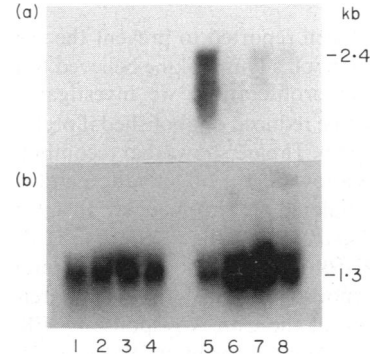

Figure 5. Northern blot of cytoplasmic mRNA extracted from unstimulated lymphocytes (lanes 1–4) or from lymphocytes stimulated with 8 μg/ml Con A for 6 hr (lanes 5–8). CsA (1 μg/ml) and FK-506 (10 nM) were added to cells for 6 hr or simultaneously with Con A. No inhibitor, lanes 1 and 5; CsA alone, lanes 2 and 6; FK-506 alone, lanes 3 and 7; both CsA and FK-506, lanes 4 and 8. The blot was sequentially probed for c-myc mRNA (a) and TcRβ mRNA (b). The size of these mRNAs is shown in kilobases.

as effective as FK-506 added together with the mitogen (Table 1). Essentially identical results were obtained when Ca^{2+} were present for the first hour of activation to allow the immediate changes in response to Con A addition to occur normally, but then chelated between 1 hr and 6 hr after mitogen addition, the period during which the drug-resistance normally develops (Table 1, line 4). The kinetics with which the activation response became resistant to the effect of FK-506, and the dependence of the development of this resistance on the presence of Ca^{2+} in the culture medium, are thus identical to the results previously reported for CsA (Kay & Benzie, 1983, 1984).

Depriving the cells of Ca^{2+} for this initial 5–6 hr period led to a reduction in the activation response measured at 24 hr. The principal reason for this is that while some early events in activation can still take place in the absence of extracellular Ca^{2+}, other key early events such as the synthesis of IL-2 cannot occur until Ca^{2+} are available, so that the response is delayed (Kay, 1987, 1989).

Interaction of FK-506 and CsA

The results reported above suggested that the effects of FK-506 on mitogen-induced lymphocyte activation were very similar to those of CsA. The effects of the two drugs in combination were therefore studied (Fig. 4). While the effects of suboptimal concentrations of the two drugs were usually observed to be additive, or in some cases even synergistic, as reported for the mixed lymphocyte reaction (Zeevi *et al.*, 1987) and activation by antigen (Sawada *et al.*, 1987), the components of the Con A (Fig. 4a) and PHA (Fig. 4b) responses that were resistant to the action of high concentrations of CsA were also unaffected by the simultaneous addition of high concentrations of FK-506.

Comparison of the effects of FK-506 and CsA on lymphocyte mRNA expression

The rapid induction of the expression of c-myc mRNA when T lymphocytes are incubated with mitogenic lectins is substantially inhibited by CsA (Reed, Nowell & Hoover, 1985). Figure 5a shows that FK-506 was similarly effective in inhibiting the induction of this mRNA by Con A.

Figure 5b shows that FK-506 and CsA also had similar effects on the expression of the constitutively expressed TcRβ mRNA. Neither agent had any significant effect on the expression of this mRNA in unstimulated cells, while in Con A-stimulated cells both increased the level of this mRNA at least two-fold (2·4-fold with FK-506 and 2·2-fold with CsA, as measured by scanning densitometry). It has been shown previously that activation of protein kinase *C* increases the expression of TcRβ mRNA, while increasing the cytoplasmic Ca^{2+} concentration has an antagonistic effect (Martinez-Valdez *et al.*, 1988; Goodier, 1989). Mitogens such as Con A provide both signals simultaneously and thus have little overall effect on the expression of this mRNA. The selective enhancement of the expression of this mRNA in the presence of Con A seen with both CsA and FK-506 is as expected for agents that block the

Table 2. Inability of IL-2 to reverse the inhibitory action of FK-506

Con A	FK-506	IL-2 (U/ml) None	25	100	200
−	−	1026±35	1728±89	2109±263	2346±174
+	−	6018±98	6498±353	6499±81	6500±242
+	+	2404±90	3226±75	3385±228	3530±125

Cultures were incubated with and without Con A and 10 nM FK-506, in the presence of the concentrations of human recombinant IL-2 indicated. Activation was assessed by determination of [^{35}S]methionine incorporation into protein at 24 hr.

Values represent mean c.p.m. incorporated per culture ±SD.

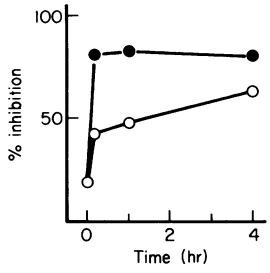

Figure 6. Reversibility of the action of FK-506. Unstimulated lymphocytes were preincubated with 1 nM FK-5(at either 4° (○) or 37° (●) for the time shown, and then collected by centrifugation and washed twice with EMEM containing 5% fetal calf serum. The cells were then incubated with or without Con A for 24 hr, and the effect of the preincubation with FK-506 on the stimulation of [^{35}S]methionine incorporation into protein determined.

effect of the Ca^{2+} signal, and further confirms the similarity of the effects of the two immunosuppressive drugs.

Effect of IL-2

As FK-506 has been reported to prevent the synthesis of IL-2 (Kino *et al.*, 1987b, c), a lymphokine believed to play a key role in T-lymphocyte proliferation, we investigated whether the inhibition would be reduced or abolished if preformed IL-2 was added to the system. Table 2 shows that recombinant human IL-2 itself induced a low degree of activation and proliferation in otherwise unstimulated pig lymphocytes. In agreement with the results of Zeevi *et al.* (1987), the response to IL-2 alone was not inhibited by FK-506 (data not shown). However, rIL-2 had little effect on the response to Con A, and its apparent partial effect on the inhibition of the Con A response by FK-506 could be almost entirely accounted for by its effect on the unstimulated cells (Table 2). Similar results were obtained when lymphokine-rich culture supernatant from pig lymphocytes activated with Con A for 24 hr was used instead of rIL-2. These results too are very similar to those obtained when CsA is used as inhibitor (Kay & Benzie, 1983, 1984).

Inhibition by FK-506 is irreversible

Preliminary experiments demonstrated that the inhibition induced by FK-506 could not readily be reversed by washing the cells free of the drug. Figure 6 shows that unstimulated lymphocytes preincubated with 1 nM FK-506 for as little as 10 min at 37° and then washed free of the drug were as strongly inhibited when subsequently stimulated with Con A as cells continuously exposed to the drug. Cells preincubated at 4° also became irreversibly inhibited, though at a slower rate.

DISCUSSION

Although the chemical structure of the macrolide FK-506 is very different from that of the cyclic endecapeptide CsA, the effects of the two immunosuppressive agents on T-lymphocyte activation by the mitogenic lectins Con A and PHA are remarkably similar. Extensive studies on the mechanism of action of CsA have led to the conclusion that the principal mechanism by which it interferes with the induction of lymphocyte proliferation is by antagonizing the Ca^{2+}-mediated signal that plays a key role in initiating this process (Kay, 1987, 1989; Klaus, 1987). FK-506 has a similar range of effects on the cells and its effects are lost with similar kinetics over the first few hours after mitogen addition, providing that Ca^{2+} were present. That part of the lectin-mediated stimulation of protein synthesis that is insensitive to inhibition by CsA is also insensitive to FK-506.

Although the action of FK-506 thus resembles that of CsA, it is generally found to show comparable effectiveness both *in vivo* and *in vitro* at concentrations two to three orders of magnitude lower. A possible explanation for this difference is suggested by our observation that the binding of FK-506 to unstimulated lymphocytes is, under the culture conditions employed, essentially irreversible. CsA presumably binds to its target much less strongly, as its effects are readily reversed by washing the cells with medium without inhibitor. The avidity of FK-506 for its target (or targets) may facilitate its (or their) identification.

While it seems clear that both FK-506 and CsA exert their effects at the same stage in the activation process, there is as yet insufficient evidence to conclude that both drugs act against precisely the same target. Indeed, FK-506 and CsA have been reported to act synergistically both in preventing transplant rejection *in vivo* (Murase *et al.*, 1987; Todo *et al.*, 1988) and in inhibition of T-lymphocyte responses *in vitro* (Zeevi *et al.*, 1987; Sawada *et al.*, 1987). As the preliminary evidence so far available suggests that at least some of the side effects of FK-506 differ from those of CsA (Thomson, 1989), combinations of the two drugs may prove to be of therapeutic value.

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