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Inhibition of T and B lymphocyte proliferation by rapamycin

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SUMMARY

The immunosuppressive macrolide rapamycin shows marked structural similarity to FK-506, and like FK-506 inhibits the activation of cultured T and B lymphocytes at concentrations as low as 10^{-10} M. However, rapamycin blocks T-lymphocyte proliferation at a much later stage than FK-506. It also inhibits human, porcine and murine T- and B-lymphocyte activation by all pathways tested, including pathways which are insensitive to FK-506, such as interleukin-2 (IL-2)-mediated proliferation of IL-2-dependent T-cell lines, activation of human peripheral blood T lymphocytes by phorbol ester and anti-CD28 and activation of murine B lymphocytes by bacterial lipopolysaccharide. Thus these two macrolides that bind competitively to the same major intracellular receptor protein inhibit T- and B-lymphocyte activation by quite distinct mechanisms.

INTRODUCTION

Rapamycin, a macrolide with anti-fungal activity produced by *Streptomyces hydroscopicus*,[1] was also found to inhibit immunological responses[2] and prevent graft rejection.[3] Interest in the immunosuppressive activity of this agent has recently been rekindled as a result of its structural resemblance to FK-506, produced by *S. tsukubaensis*, which is strongly immunosuppressive in experimental systems, inhibits the activation of cultured T and B lymphocytes in response to some mitogenic signals, and has now entered clinical trial as an immunosuppressive drug for use in organ transplantation.[4-8]

Rapamycin has been reported to inhibit the activation of murine T lymphocytes *in vitro*.[9-11] We here report a detailed comparison of the effects of rapamycin on the activation of cultured human, murine and porcine T and B lymphocytes by a range of mitogenic stimuli with those of FK-506 and cyclosporin A (CsA).

Our previous studies have established that the sensitivity of lymphocyte activation to inhibition by FK-506 or CsA varies with the nature of the mitogenic signal used. In brief, those responses, or aspects of responses, that are dependent on the elevation of the cytoplasmic Ca^{2+} concentration are blocked by either FK-506 or CsA, while responses that are mediated by protein kinase C activation alone or are otherwise Ca^{2+}-independent are not sensitive to either of these drugs.[6,12-15] Similar conclusions have been reached for B lymphocytes. Activation by bacterial lipopolysaccharide (LPS) is Ca^{2+}-independent and unaffected by FK-506 or CsA, while activation by anti-mIgM is dependent on a Ca^{2+} signal and strongly inhibited by both agents.[16,17]

Abbreviations: anti-mIgM, goat anti-mouse IgM; Con A, concanavalin A; CsA, cyclosporin A; IL-2, interleukin-2; LPS, lipopolysaccharide from *Salmonella typhi*; PHA, phytohaemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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MATERIALS AND METHODS

Materials

Rapamycin was kindly provided by Dr Joseph Chang, Wyeth-Ayerst Research Laboratories, Princeton, NJ, and FK-506 by the Fujisawa Pharmaceutical Company, Osaka, Japan. Both agents were dissolved in ethanol to give 1 mM stock solutions, which were stable for at least several months when stored at −20°. Ethanol was added to control cultures at concentrations that did not exceed 0·4% and were without effect on the responses studied. Concanavalin A (Con A; Flow Laboratories, Irvine, Ayrshire, U.K.), Phytohaemagglutinin (PHA-P; Difco Laboratories, Detroit, MI) and lipopolysaccharide (LPS; from *S. typhi*, Sigma Laboratories, Poole, Dorset, U.K.) were dissolved in tissue culture medium. The anti-CD3 monoclonal antibody OKT3 and the anti-CD28 monoclonal antibody 9.3 were kindly provided by Drs Keith Moore (Tenovus Laboratory, Southampton, U.K.) and Jeffrey Ledbetter (Oncogen Corp., Seattle, WA). Goat anti-mouse IgM (μ-chain specific) was purchased from Sigma Laboratories.

Cell culture

Human and porcine peripheral blood lymphocytes and spleen cells from male CBA mice were prepared and cultured as described previously[13,17] and their activation assessed by determination of the rate of [^{35}S]methionine incorporation into protein or [^{3}H]thymidine incorporation into DNA during a terminal 4-hr pulse, as described previously.[18] One microcurie of radioisotope was added to each of three or more replicate

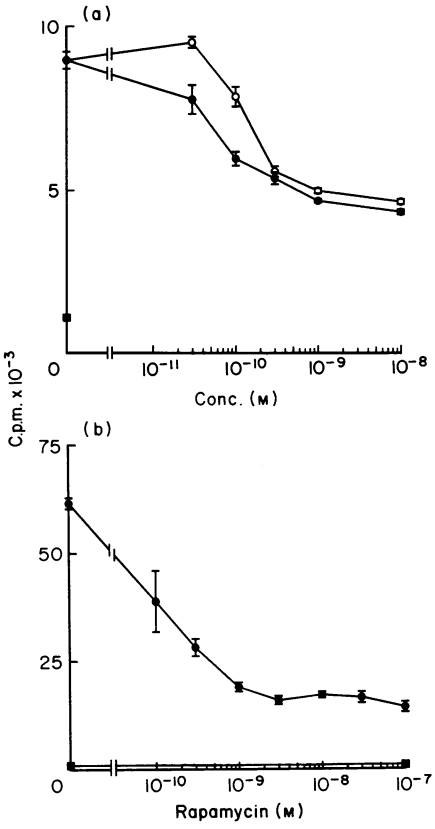

Figure 1. Effect of rapamycin and FK-506 on the activation of porcine peripheral blood lymphocytes by Con A. Lymphocytes were incubated with 5 μg/ml Con A and the concentrations of rapamycin (●) and FK-506 (○) indicated. (■) Unstimulated. Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 24 hr (a) or the rate of incorporation of [^{3}H]thymidine into DNA at 48 hr (b).

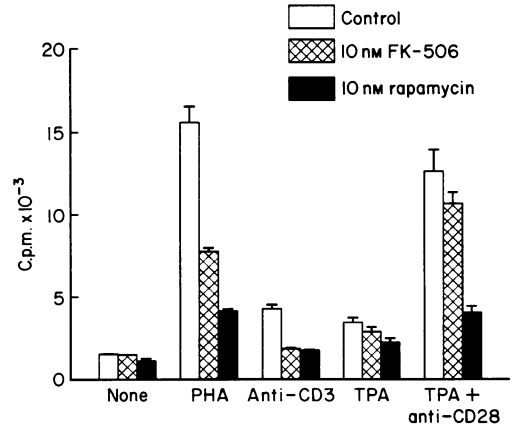

Figure 2. Comparison of the effects of rapamycin and FK-506 on the activation of human peripheral blood lymphocytes. Lymphocytes were incubated without mitogen; with 10 μg/ml PHA; 2·5 ng/ml anti-CD3; 20 ng/ml TPA; or 20 ng/ml TPA + 1 μg/ml anti-CD28. Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 46 hr.

cultures. Mean values ± SD are shown, except where the SD lies within the space occupied by the symbol.

RESULTS

The effects of rapamycin on T- and B-lymphocyte responses to mitogen

Like FK-506, rapamycin strongly but incompletely inhibited the response of porcine peripheral blood lymphocytes to activation by mitogenic lectins such as Con A (Fig. 1). Highly significant inhibition of the lectin-induced stimulation of protein synthesis (37 ± 8% inhibition, 16 experiments) was observed with 0·1 nM rapamycin and inhibition was approaching maximal (52 ± 4%, 17 experiments) at 1 nM rapamycin. However, the inhibition was incomplete, with even 100 nM concentrations having little additional effect on the rapamycin-insensitive component of the Con A response (59 ± 4% inhibition, seven experiments). The effective concentrations of rapamycin were marginally lower than the FK-506 concentrations required to achieve a comparable effect (Fig. 1a) and about three orders of magnitude lower than for CsA. However, rapamycin present over a 24 hr period also caused a reproducible inhibition of the rate of protein synthesis by unstimulated lymphocytes. Highly significant inhibition compared to solvent controls (18 ± 4%, six experiments) was observed with 0·1 nM rapamycin, and again there was no further increase in the extent of the inhibition as the rapamycin concentration was raised to 100 nM (19 ± 5% inhibition, eight experiments). By comparison, FK-506 was without significant effect when added to unstimulated lymphocytes for 24 hr (4 ± 5% inhibition in the first 25 comparisons using 10 nM FK-506.[6]

The spectrum of rapamycin activity against other mitogen responses was quite different from those of FK-506 and CsA. Lymphocyte proliferation in response to all mitogenic signals studied was inhibited (although some early events in activation that are sensitive to FK-506 and CsA were unaffected by rapamycin, as previously reported by Dumont *et al.*).[9] Thus rapamycin inhibited the FK-506-sensitive responses of human lymphocytes to lectins and anti-CD3, but also blocked the FK-506- and CsA-resistant response to co-stimulation with the phorbol ester TPA and anti-CD28 (Fig. 2). Rapamycin inhibited the responses of porcine peripheral blood lymphocytes to the lectins Con A and PHA to equivalent extents (data not shown), though the Con A response is considerably more sensitive to FK-506 and CsA.[6,19] Similarly, the FK-506-insensitive stimulation of peripheral blood lymphocyte protein synthesis by TPA was as sensitive to inhibition by rapamycin as the FK-506-sensitive response to Con A (Fig. 3). The proliferative response of an IL-2-dependent porcine T-cell line to IL-2 was strongly inhibited by 0·01 nM rapamycin, though resistant to FK-506 at concentrations four orders of magnitude higher (Fig. 4).

The conclusion that the spectrum of activity of rapamycin differs from those of FK-506 and CsA was further supported by experiments using cultured CBA mouse spleen cells. The CsA- and FK-506-sensitive B-cell response to anti-mIgM was inhibited by rapamycin, but the CsA- and FK-506-resistant response of B cells to LPS was also sensitive to rapamycin (Fig. 5). The response to all concentrations of both B-cell mitogens was sensitive to rapamycin (Fig. 5), and the concentrations of rapamycin required to inhibit the LPS response were no higher

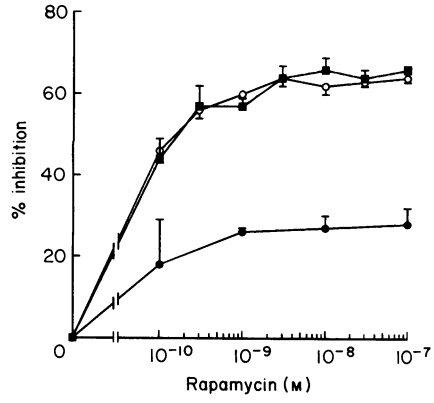

Figure 3. Effect of rapamycin on the activation of porcine lymphocytes by TPA. Lymphocytes were incubated without mitogen (●), with 5 μg/ml Con A (○) or with 10 ng/ml TPA (■). Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 24 hr, and the percentage inhibition by rapamycin calculated. Control values were 1630 c.p.m. for unstimulated cultures, and an additional 17,552 c.p.m. for Con A-stimulated cultures and 3778 c.p.m. for TPA-stimulated cultures.

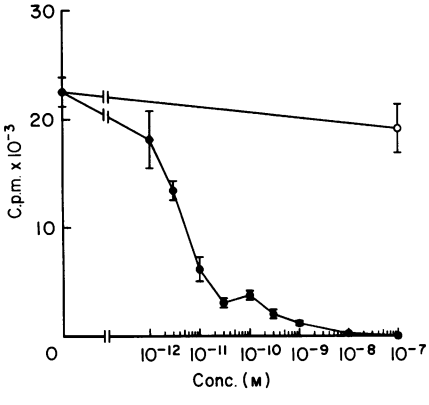

Figure 4. Effect of rapamycin (●) and FK-506 (○) on IL-2-driven proliferation of a porcine IL-2-dependent T-cell line. Cells at 2×10^4/ml were cultured with 50 U/ml recombinant human IL-2 and activation was assessed by determination of the rate of [^{3}H]thymidine incorporation into DNA at 48 hr.

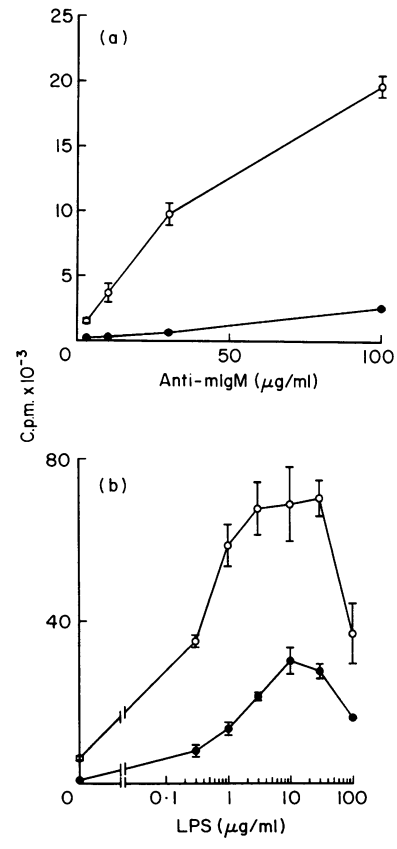

Figure 5. Effect of rapamycin on the response of murine spleen cells to anti-mIgM (a) or LPS (b). Cells were incubated with (●) or without (○) 10 nM rapamycin and activation was assessed by determination of the rate of incorporation of [^{3}H]thymidine into DNA at 70 hr.

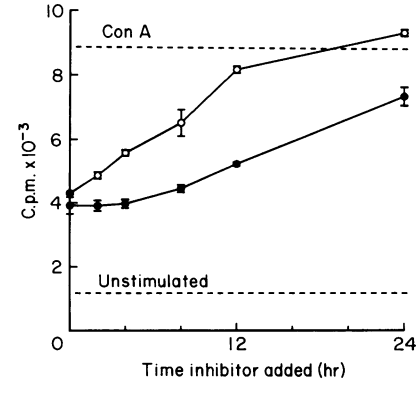

Figure 6. Effect of delayed addition of rapamycin and FK-506. Porcine lymphocytes were incubated with 10 μg/ml Con A and 10 nM rapamycin (●) or FK-506 (○) added at the times indicated thereafter. Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 28 hr.

than those required to inhibit the response of CBA spleen cells to Con A (results not shown).

Even responses inhibited by both rapamycin and FK-506, such as the activation of peripheral blood lymphocytes by Con A, were sensitive to the two drugs at different stages (Fig. 6). As previously reported, sensitivity to FK-506 and CsA is progressively lost over the initial 8–12 hr after mitogen addition.[6,19] However, rapamycin was equally effective when added together with or up to 4 hr after mitogen addition, and sensitivity declined only slowly thereafter. Thus while the key CsA- and FK-506-sensitive event in activation is completed within the first few hours after mitogen addition, the rapamycin-sensitive step occurs later in the response. This conclusion is in good agreement with previous studies investigating the effects of these agents on murine spleen cells co-stimulated with TPA and Ca^{2+}ionophore or Con A.[9,11]

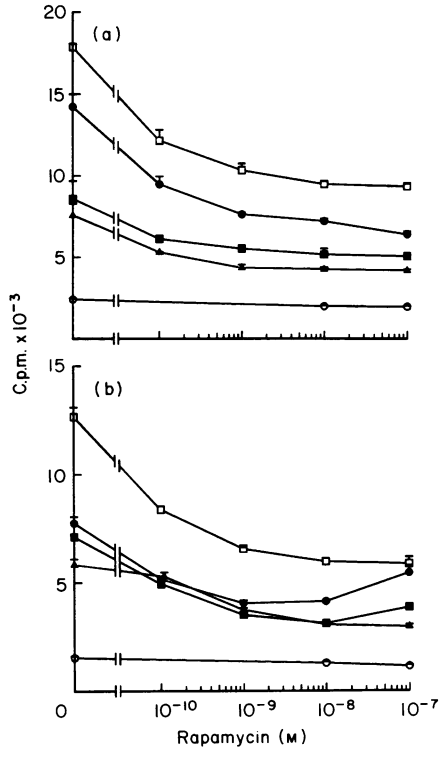

Figure 7. Interaction rapamycin and with CsA (a) and FK-506 (b). Porcine lymphocytes were incubated without mitogen (○) or with 5 μg/ml Con A (□). Where indicated 30 nM (●), 100 nM (■) or 1 μM (▲) CsA (a) or 1 nM (●), 10 nM (■) or 100 nM (▲) FK-506 (b) and rapamycin at the concentrations shown were added together with Con A. Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 24 hr.

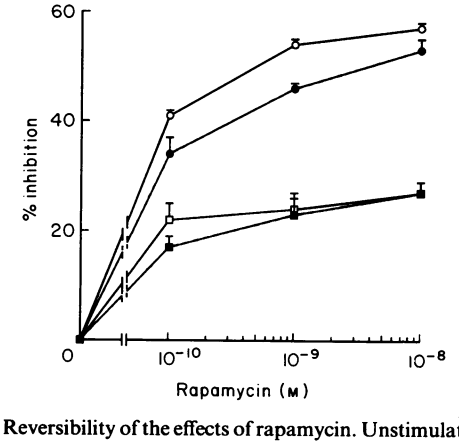

Figure 8. Reversibility of the effects of rapamycin. Unstimulated porcine lymphocytes were incubated with the concentrations of rapamycin indicated for 1 hr at 37°. Some cells (solid symbols) were then washed three times with 20 ml fresh culture medium to remove unbound rapamycin, a procedure previously shown to cause the effective removal of CsA. In other cultures rapamycin was present throughout (open symbols). Cells were then cultured with (○,●) or without (□,■) 5 μg/ml Con A. Activation was assessed by determination of the rate of incorporation of [^{35}S]methionine into protein at 24 hr and the percentage inhibition of unstimulated incorporation and of the Con A response by rapamycin calculated.

Interaction of rapamycin with FK-506 and CsA

Rapamycin, FK-506 and CsA all cause incomplete inhibition of lectin-induced T-lymphocyte activation. We have previously reported that the effects of high concentrations of CsA and FK-506 are not additive, indicating that both drugs inhibited the same component of the overall response.[6] We therefore investigated the effects of rapamycin when added in combination with CsA or FK-506 (Fig. 7).

Figure 7a shows that the results obtained when Con A-stimulated peripheral blood lymphocytes were exposed simultaneously to rapamycin and CsA were quite different from those obtained previously with FK-506 and CsA. The effects of rapamycin and CsA appeared independent, in that each was still able to cause a similar degree of inhibition of the response to Con A whether the other was absent or present at maximally effective concentrations. These results confirm the conclusion reached above that the two agents act independently at different steps, but there was no indication that they acted synergistically or that they inhibited reciprocal aspects of the response.

The results obtained when rapamycin and FK-506 were combined were more complicated (Fig. 7b). Again, combination of equimolar concentrations of the two agents gave considerably greater inhibition of the Con A response than could be achieved with maximally effective concentrations of either alone, confirming that rapamycin also acts in a different way from FK-506. However, apparently anomalous results were obtained when either macrolide was present in marked molar excess over the other. When 1 nM FK-506 was combined with 100 nM rapamycin the inhibition observed was significantly less than when both were present at 1 nM, and little greater than seen with 100 nM rapamycin alone. Similarly, 0·1 nM rapamycin caused significant inhibition when added alone or together with 1 nM or 10 nM FK-506, but caused much less inhibition than expected when combined with 100 nM FK-506. Similar apparent two-way competition between rapamycin and FK-506 has been observed in murine spleen cells.[10] The probable explanation is that despite their very different actions at the cellular level, rapamycin and FK-506 both bind competitively to the same cytoplasmic binding protein FKBP.[20] The demonstration of competition at both the binding protein and the functional levels strongly favours the hypothesis that FKBP mediates the biological effects of both FK-506 and rapamycin.

Uptake of rapamycin by lymphocytes

We have previously shown that the action of FK-506 at the cellular level is effectively irreversible, in marked contrast to that of CsA which can be quite readily removed by washing.[6] Figure 8 shows that unstimulated peripheral blood lymphocytes preincubated with rapamycin for 1 hr at 37° and then washed extensively showed an inhibition of their response to Con A added after washing away unbound inhibitor that was almost as strong as when the inhibitor was present throughout. The inhibition of unstimulated lymphocyte protein synthesis observed after 24 hr was also of similar magnitude whether the lymphocytes were preincubated with rapamycin for 1 hr or the drug was present for the entire 24 hr. Rapamycin, like FK-506, is thus rapidly taken up and strongly retained by cultured cells. The rapamycin concentration required to inhibit the Con A

response increases as a function of the cell concentration (results not shown). Indeed, the remarkable rapamycin sensitivity of the IL-2 response of the porcine T-lymphoid line seen in Fig. 4 above, with 50% maximal inhibition observed with less than 5 pM rapamycin, is probably due more to the low cell concentration employed in this assay than to any particular sensitivity of the IL-2 response to the drug.

DISCUSSION

The results reported above show that there are some superficial similarities between the biological actions of rapamycin and FK-506, in that both inhibit, at concentrations below 10^{-9} M and to comparable extents, the activation of T lymphocytes by anti-CD3 or lectins such as Con A and the activation of B lymphocytes from murine spleen by anti-mIgM, and that both bind to lymphocytes rapidly and effectively irreversibly. However, it is clear that while the actions at the cellular level of FK-506 are very similar to those of CsA, rapamycin acts at a quite different and later stage in the activation process. This thus confirms and extends to new species and lymphocyte activation pathways the conclusion drawn previously from studies with T lymphocytes from murine spleen by Dumont *et al.*[9] and Metcalfe & Richards.[11]

However, rapamycin has structural homology with FK-506 and both its binding to FKBP[20] and its effects at the cellular level (Fig. 7b)[10] are competitive with FK-506. CsA has no evident structural homology with the two macrolides and binds to a quite distinct binding protein, albeit one which shares with FKBP peptidyl-prolyl cis-trans isomerase activity.[20-23] This paradox remains to be resolved. The possibility that the immunosuppression caused by rapamycin, FK-506 and CsA is mediated by some as yet unidentified minor binding proteins distinct from FKBP and cyclophilin cannot yet be altogether ruled out, though such hypotheses would have to account for the reciprocal competition between the two macrolides. An alternative possibility would be that the inhibition of lymphocyte activation might result from the inactivation by drug-complexed peptidyl-prolyl isomerase of key target proteins, perhaps substrates, with which they interacted. On such a hypothesis rapamycin- and FK-506-complexed FKBP would have to inactivate different target proteins.

The effective irreversibility at the cellular level of the effects of the macrolides rapamycin and FK-506 is also worthy of note. If this observation can be extrapolated to the *in vivo* situation, it raises the possibility that intermittent periods of low drug level might have less serious consequences than if the effects of the drug were more readily reversed. In addition the ability of rapamycin to act at later stages in lymphocyte activation and antagonize activation by a wider spectrum of activation pathways may prove of therapeutic value.

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