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Spleen lymphocyte populations and expression of activation markers in rats treated with the potent new immunosuppressive agent FK-506

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SUMMARY

Rats were immunized systemically with sheep red blood cells (SRBC) and treated with either FK-506 (1 mg/kg/day) or cyclosporin A (CsA) (25 mg/kg/day) for 7 days. Profound (>90%) suppression of the production of splenic IgM-secreting plasma cells and circulating antibody levels was observed in animals receiving either drug. Immunosuppression was accompanied by significant increases in the incidence and absolute numbers of $OX8^+$ (T-cytotoxic/suppressor) lymphocytes in the spleen, and there were corresponding reductions in the $W3/25^+ : OX8$ ($CD4^+ : CD8^+$) ratio. The magnitude of these changes was not affected by drug combination. There were no significant alterations in B cells with either agent, whilst a small but significant increase in the incidence of macrophages was observed in all drug-treated groups. Neither FK-506 nor CsA affected IL-2 receptor (OX39) or MHC class II (OX6) antigen expression. This study demonstrates the remarkable immunosuppressive potency of FK-506 and its underlying capacity, like CsA, to affect regulatory T-lymphocyte subsets *in vivo*.

Considerable interest has been generated recently in the immunosuppressive properties of a newly discovered macrolide antibiotic, FK-506, which is isolated from the fermentation broth of a soil fungus, *Streptomyces tsukubaensis* (Kino *et al.*, 1987a; Ochiai *et al.*, 1987c). In animals, FK-506 exhibits potent inhibitory effects on cell-mediated and humoral immunity (Kino *et al.*, 1987a, Inamura *et al.*, 1988) and significantly prolongs organ allograft survival (Murase *et al.*, 1987; Ochiai *et al.*, 1987b; Lim, Thiru & White, 1987). Like the fungal metabolite cyclosporin A (CsA), FK-506 inhibits interleukin production, mixed lymphocyte reactivity and the generation of cytotoxic T cells (Kino *et al.*, 1987b; Thomson & Webster, 1988). Compared with CsA, however, these anti-lymphocytic effects are achieved at considerably (up to several hundred-fold) lower concentrations (Kino *et al.*, 1987b; Zeevi *et al.*, 1987). There is little evidence of toxicity in rats (Nalesnik *et al.*, 1987), although certain pathological effects have been described in dogs and baboons (Ochiai, Hamaguchi & Isono, 1987a; Thiru, Collier & Calne, 1987). Equally significant are reports that subtherapeutic doses of FK-506 and CsA can act synergistically to prolong allograft survival (Murase *et al.*, 1987) and inhibit lymphocyte responses *in vitro* (Zeevi *et al.*, 1987). To date, there is no published information concerning the influence of FK-506 on immunoregulatory lymphocyte populations either *in vivo* or *in vitro*. This study therefore, was instigated to examine the influence of FK-506 and CsA, administered either alone or together, on rat spleen cell populations and the expression of lymphocyte activation-associated antigens.

Groups of six male Sprague–Dawley rats (Charles River, Margate, Kent) with a mean initial body weight of 250 g were used. They were immunized intraperitoneally on Day 0 with 10^9 sheep red blood cells (SRBC; Difco Laboratories, West Molesey, Surrey) in 1 ml sterile phosphate-buffered saline, pH 7·2. FK-506 (Lot 011050L, Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan) was dissolved in absolute methanol, to which was added nine parts olive oil. After thorough mixing, the methanol was evaporated off at 60° (10 min). The resulting FK-506 solution was injected (0·1 ml) intramuscularly (1 mg/kg/day) into one of the four limbs, with daily rotation. Cyclosporin A (CsA, batch 83601; Sandoz Ltd, Basle, Switzerland) was prepared in 10% ethanol in olive oil (25 mg/ml) and administered by gavage (25 mg/kg/day) using a No. 4 fine-gauge intravenous cannula (Portex Ltd., Hythe, Kent). FK-506 and CsA were administered from Day 0 to 6, inclusive. On Day 7, blood was collected into plain tubes from cleaned tail tips under ether anaesthesia then serum separated and stored (−20°) for antibody titration. The animals were killed, spleens removed, cell suspensions prepared and mononuclear cells isolated as detailed elsewhere (Webster & Thomson, 1987). An indirect immunofluorescence method was employed to demonstrate cell phenotypes, using appropriate mouse anti-rat monoclonal antibodies (Serotec Ltd, Bicester, Oxon) at the following final dilutions: W3/25 ($CD4^+$ helper/inducer T cells, macrophages), 1 : 500; OX8 ($CD8^+$ cytotoxic/suppressor T cells, natural killer

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Table 1. Effects of FK-506 and CsA on IgM-producing plasma cells and circulating antibody titres

Treatment (dose)	PFC/10^6 spleen cells	% reduction	Serum total HA titre ($-\log_2$)
Vehicle	177·3±90·6	0	8·9±1·5
FK-506 (1 mg/kg)	2·2±2·1*	98	3·6±0·9*
CsA (25 mg/kg)	14·2±8·8*	92	2·4±0·6*
FK-506+CsA	13·0±5·0*	93	3·0±0·0*

Results are means±1 SD determined 7 days after immunization.
* $P<0·001$ compared with vehicle-treated controls.

cells), 1:400; OX12 (κ light chains, pan B cells), 1:500; ED1 (monocytes/macrophages), 1:500; OX39 (IL-2 receptor), 1:300 and OX6 (MHC class II), 1:300. On receipt, each antibody was diluted 1:20 in PBS, containing 1% w/v bovine serum albumin (BSA; Sigma Chemical Company Ltd, Poole, Dorset) and 0·1% w/v sodium azide, before storage in aliquots at −70°. Immediately before use, the antibodies were diluted further in PBS containing 1% v/v, heat-inactivated, pooled normal goat serum (SAPU, Carluke, Lanarkshire). The secondary antibody was FITC-conjugated goat IgG anti-mouse IgG (Becton-Dickinson Ltd, Cowley, Oxon), which was pre-incubated for 45 min at 4° in PBS, containing 10% normal rat serum. The staining procedure and further details of analysis using an 'EPICS C' flow cytometer (Coulter Electronics, Luton, Beds) have been published elsewhere (Webster & Thomson, 1987).

Single antibody (IgM)-producing plasma cells in spleen cell suspensions were estimated by the plaque assay of Cunningham & Szenberg (1968). Serum total haemagglutinin titres to SRBC were determined on heat-inactivated samples as described by Hudson & Hay (1980). The significance of differences between means was calculated using the Student's *t*-test for independent means.

The immunosuppressive properties of FK-506 and CsA are shown in Table 1. Profound inhibition of both the splenic plaque-forming cell (PFC) response and circulating antibody titres were observed with either drug alone and with both agents in combination. Flow cytometric analysis of splenic mononuclear cells (Table 2) revealed similar incidences and absolute numbers of W3/25$^+$ cells in all groups. In contrast, significant increases of similar magnitude in OX8$^+$ cells were observed in both FK-506- and CsA-treated animals, although there was no further increase with drug combination. These changes were reflected, in all drug-treated groups, in corresponding, significant reductions ($P<0·01$) in the W3/25$^+$: OX8$^+$ ratio. Whilst neither immune suppressant alone affected numbers of splenic OX12$^+$ (B) cells, a small but statistically significant increase in macrophages with FK-506 or CsA was recorded. Compared with vehicle control values, there was an apparent increase ($P<0·05$) in B cells but no change in macrophages in the drug combination group. No significant effect of either agent on the expression of MHC class II antigens or IL-2 receptors was observed.

These findings confirm recent reports of the potent immunosuppressive activity of FK-506 in the rat and other laboratory species. In addition, however, we have shown that this activity is accompanied by significant increases in the proportion and absolute numbers of immunoregulatory T-suppressor/cytotoxic (OX8$^+$) cells within the spleen—a property shared with CsA. At least with CsA, the reduction in the CD4$^+$: CD8$^+$ ratio appears to be antigen-dependent, since in this laboratory similar changes have not been observed in the spleens of unimmunized rats after 7 days treatment. The augmentation of T-suppressor cell numbers by FK-506 is in keeping with evidence that unresponsiveness to cardiac allografts in rats treated with this agent can be adoptively transferred to naive recipients using spleen cells (Ochiai *et al.*, 1987b). Whilst there is evidence that FK-506 and CsA can act synergistically to suppress functional T-cell responses (Zeevi *et al.*, 1987; Murase *et al.*, 1987), we observed no further reductions in the T-helper/suppressor cell ratio with combined drug administration. This latter finding may simply

Table 2. Effects of FK- 506 and CsA on mononuclear cell subsets and the expression of activation antigens

Treatment	W3/25$^+$	OX8$^+$	OX12$^+$	ED-1$^+$	OX6$^+$	OX39$^+$	W3/25$^+$ / OX8$^+$
Vehicle							
% positive cells	22·6±5·3	13·2±3·4	54·3±7·0	1·1±0·7	54·6±5·4	1·7±0·8	1·7±0·3
Positive cells per spleen ($\times 10^7$)	12·7±4·4	7·5±2·9	30·4±6·3	0·7±0·5	31·0±7·6	1·0±0·7	—
FK-506							
% positive cells	26·3±3·5	21·8±3·9***	52·1±7·6	2·5±1·3*	53·1±6·6	2·9±1·5	1·1±0·2**
Positive cells per spleen ($\times 10^7$)	14·5±2·8	12·9±2·3**	31·1±4·5	1·5±0·8*	31·6±3·9	1·7±0·9	—
CsA							
% positive cells	27·9±4·9	23·2±4·2***	61·1±7·2	3·2±1·2***	58·6±5·7	3·0±1·5*	1·2±0·2**
Positive cells per spleen ($\times 10^7$)	14·3±7·3	12·1±5·4*	32·1±14·0	1·8±1·1*	30·9±13·1	1·7±1·3	—
FK506+CsA							
% positive cells	23·1±7·9	20·9±7·4**	56·8±5·1	3·6±1·7**	56·3±4·5	3·0±1·9	1·1±0·2**
Positive cells per spleen ($\times 10^7$)	16·2±5·5	14·6±5·2**	39·6±3·5*†	2·5±1·2**	39·3±3·1	2·1±1·4	—

Results are means±1 SD.
* $P<0·05$; ** $P<0·01$; *** $P<0·001$ compared to vehicle controls.
† $P<0·02$ compared to FK-506 alone.

reflect the highly immunosuppressive effect of either drug when administered alone at the dosage selected in this study.

It has been suggested that FK-506 may suppress IL-2 receptor expression on T cells *in vitro* (Kino *et al.*, 1987a), but we observed no evidence of this in the present *in vivo* study. Failure of CsA to affect IL-2 receptor expression (either alone or in combination with FK-506) is in keeping with the findings of most but not all authors concerning the drugs's action on this lymphocyte activation marker (Thomson & Webster, 1988).

FK-506 is clearly a very powerful immunosuppressive agent with significant potential, both as an analytical tool in cellular immunology and as a therapeutic agent. In the latter context, progress towards clinical evaluation will depend on the outcome of further toxicological studies, especially those in non-human primates.

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