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Immunosuppressive activity of FK-506 in rats: flow cytometric analysis of lymphocyte populations in blood, spleen and thymus during treatment and following drug withdrawal

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SUMMARY

Rats were immunized systemically with sheep red blood cells (SRBC) and given either FK-506 (1 mg/kg) or drug vehicle by i.m. injection for 7 days. In animals receiving FK-506, there was suppression (87%) of the splenic plaque-forming cell response on day 4 and marked reductions in the serum antibody titre throughout the 3-week period following immunization. Sequential flow cytometric analyses of blood lymphocytes revealed statistically significant attenuation, by FK-506, of the increase in relative numbers of OX-12$^+$ (B) cells between days 4 and 7. Following drug withdrawal, OX-12 values remained elevated, whereas in control animals a decline was observed. These changes were reflected in concomitant increases in the relative numbers of OX-19$^+$ (CD3$^+$), W3/25$^+$ (CD4$^+$) and OX-8$^+$ (CD8$^+$) T cells; however, due to an overall reduction in lymphocytes by day 7, absolute values were not significantly affected compared with controls. The pattern of changes in OX-6$^+$ (MHC class II$^+$) cells in blood was similar to that observed for B cells. FK-506 also suppressed increases in the small proportion of blood-borne OX-40$^+$ (activated CD4$^+$) cells and OX-39$^+$ (interleukin-2 receptor$^+$) cells in the 7 day period following immunization; thereafter values for activation marker expression between treatment and control groups were similar. In the spleen, there were fewer significant differences between FK-506 and control groups in the incidences of cells expressing the above markers. OX-8$^+$ cells, however, were significantly higher in drug-treated animals on day 7, and there were also reductions in the small proportions of OX-39$^+$ and OX-40$^+$ cells when compared with controls. In the thymus, reversible medullary atrophy induced by FK-506 was accompanied on day 7 by increases in the incidence of CD4$^+$ and CD8$^+$ cells and by a concomitant reduction in OX-44$^+$ mature, medullary thymocytes. Two weeks after drug withdrawal, the phenotypic marker expression profile had been restored to normal in blood, spleen and thymus. These data provide new information on the apparent capacity of FK-506 to interfere with T cell maturation and its influence on lymphocyte activation *in vivo*.

Keywords FK-506 lymphocytes blood spleen thymus

INTRODUCTION

Cyclosporin A (CyA) has become the agent of choice in the immunotherapy of allograft rejection, but its clinical potential, including its role in the treatment of certain autoimmune disorders, is restricted by various side effects, in particular nephrotoxicity. FK-506 is a recently discovered macrolide antibiotic, isolated from the fungus *Streptomyces tsukubaensis*, which shares many of the anti-lymphocytic and immunosuppressive properties of CyA (reviewed by Thomson, 1989). These include selective inhibition of the early phase of CD4$^+$ T cell activation and lymphokine production (Kino *et al.*, 1987a, b; Yoshimura *et al.*, 1989b), suppression of cytotoxic T cell generation (Kino *et al.*, 1987b; Yoshimura *et al.*, 1989a), the apparent sparing of suppressor cells (Ochiai *et al.*, 1987b; Woo, Stephen & Thomson, 1988) and the capacity of short courses of the drug to induce tolerance to alloantigens in the rat (Ochiai *et al.*, 1987b). However, FK-506 has been shown to be considerably more potent than CyA, both *in vitro* and *in vivo*. Moreover, FK-506 is reported to potentiate the uptake of CyA by human lymphocytes (Sanghvi *et al.*, 1987b) and to act synergistically with CyA to inhibit mixed lymphocyte reactivity (Zeevi *et al.*, 1987) and organ allograft rejection (Murase *et al.*, 1987; Todo *et al.*, 1987). Differential toxicity of FK-506 has been observed in non-human species (Collier, Thiru & Calne, 1987; Ochiai *et al.*,

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1987a; Stephen *et al.*, 1989; Thiru, Collier & Calne, 1987), with no serious side effects reported in a recent study of baboons (Todo *et al.*, 1988).

These findings suggest that FK-506 may prove useful as a clinical immunosuppressive agent, perhaps as an adjunct to established forms of therapy. However, little is known as yet about the effects of FK-506 on the immune system. In this study, we have examined sequential changes in functional lymphocyte populations and activation antigen expression in blood, spleen and thymus of immunized animals during and after a short course of administration of the drug.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (with a mean initial body weight of 235 g) were purchased from Charles River (Margate, UK) and maintained in the University Animal Department, Foresterhill, Aberdeen on CRM diet (Labsure Animal Foods, K. & K. Greeff, Croydon, UK) with tap water *ad libitum*. FK-506 (Fujisawa Pharmaceutical Co., Osaka, Japan; Lot 011504) was provided in powder form and dissolved in absolute methanol. A 10% solution of the FK-506 containing methanol in olive oil (Boots, Nottingham, UK) was prepared, the methanol evaporated off at 60°C for 5 min and the solution injected (0·1 ml; 1 mg/kg per day) intramuscularly into one of the four limbs, with daily rotation.

Immunization

Sheep red blood cells (SRBC) obtained from sheep blood in Alsever's solution (Difco Laboratories, West Molesey, UK) were washed three times in ice-cold Dulbecco 'A' phosphate-buffered saline (PBS), pH 7·2, and 10^9 in 1 ml administered by a single i.p. injection.

Experimental protocol

Groups of six rats were immunized on day 0 and treated with FK-506 from days 0 to 6, inclusive. A drug vehicle-treated control group was investigated in parallel. Standard haematological tests and investigations of antibody titres, performed on samples of tail blood, were undertaken immediately before immunization (day 0) and on days 4, 7, 14 and 21. At the same time-points, groups of animals were killed by terminal ether anaesthesia. Blood (removed by cardiac puncture), spleens and thymuses were taken for immunophenotypic analysis.

Preparation of cell suspensions

Mononuclear leucocytes (MNL) were isolated from blood anticoagulated with acid citrate dextrose by centrifugation over Ficoll–Isopaque. Suspensions of splenic MNL or thymocytes were prepared as previously described (McIntosh *et al.*, 1986). Contaminating red cells were lysed in 0·83% (w/v) NH_4Cl, pH 7·2. The washed cells were resuspended at 10^7/ml in RPMI-1640 with 10% fetal calf serum (FCS) (Gibco, Paisley, UK).

Immunofluorescence staining

An indirect immunofluorescence method was used to demonstrate cell phenotypes, using appropriate primary mouse anti-rat monoclonal antibodies (Serotec, Bicester, UK) at the following dilutions: OX-19 ($CD3^+$ T cells) 1:500; W3/25 ($CD4^+$ helper/inducer T cells, macrophages) 1:500; OX-8 ($CD8^+$ cytotoxic/suppressor T cells, natural killer cells), 1:400; OX-7 (thy 1.1^+ T cells) 1:250; OX-44 (mature medullary thymocytes) 1:500; OX-40 (activated $CD4^+$ cells, a gift from Dr A. F. Williams) 1:400; OX-12 (κ light chains, *pan*-B cells) 1:500; ED1 (monocytes/macrophages), 1:500; OX-18 (MHC class I) 1:500; OX-6 (MHC class II) 1:300; OX-39 (interleukin-2 receptor; IL-2R), 1:300. On receipt, each antibody was diluted 1:20 in PBS, containing 1% w/v bovine serum albumin (BSA; Sigma Chemical Company, Poole, UK) and 0·1% w/v sodium azide, before storage in aliquots at −70°C. Immediately before use, the antibodies were further diluted in PBS containing 1% v/v heat-inactivated, pooled normal goat serum (Scottish Antibody Production Unit, Carluke, UK). The secondary antibody was FITC-conjugated goat IgG anti-mouse IgG (Becton-Dickinson, Cowley, UK), which was pre-incubated for 45 min at 4°C in PBS, containing 10% normal rat serum. The staining procedure and further details of lymphocyte analysis using an 'EPICS C' flow cytometer (Coulter Electronics, Luton, UK) have been published elsewhere (Webster & Thomson, 1988).

Table 1. Effects of FK-506 on humoral immunity

Treatment	PFC/10^6 spleen cells on day 4 (% relative to control)	Day after immunization (−$\log_2$ total HA titre) 4	7	14	21
Control	155·6±68·4 (100%)	6·3±2·5	7·3±1·7	7·5±0·6	7·8±0·5
FK-506	20·9±10·9* (13·4%)	4·3±1·6	4·8±1·0	4·8±0·4†	4·0±1·1†

Results are means±1s.d. obtained from groups of six rats.

FK-506 (1 mg/kg) or drug vehicle was administered i.m. on days 0–6, inclusive.

PFC, plaque-forming cells; HA, haemagglutination.

* $P<0·05$; † $P<0·001$ compared with vehicle-treated controls.

Antibody-producing cells and circulating antibody

Single, antibody (IgM) producing plasma cells in spleen cell suspensions were estimated by the plaque assay of Cunningham & Szenberg (1968). Total haemagglutinin titres to SRBC in heat-inactivated serum samples were determined by standard procedures, using a Titertek multiple diluter and *U*-shaped, 96-well plates.

Statistical analysis

The significances of difference between means were determined using Student's *t*-test for dependent or independent means, as appropriate.

RESULTS

Immunosuppressive activity of FK-506

The humoral immune response to SRBC in rats given FK-506 (1 mg/kg i.m.) on days 0–6 relative to immunization on day 0, is shown in Table 1. There was impairment (87%) of the primary splenic IgM plaque-forming cell (PFC) response on day 4, together with marked depression of total serum haemagglutinin titres both during (day 4) and in the two week period subsequent

Table 2. Effect of FK-506 on numbers of blood, spleen and thymus mononuclear cells

Treatment	Day after immunization				
	0	4	7	14	21
Blood ($\times 10^9/l$)					
Control	9·6±3·7	16·1±4·5	17·0±0·6	19·9±0·4	13·7±4·5
FK-506	—	15·2±2·8	12·9±2·0*	18·8±6·7	11·0±2·0
Spleen ($\times 10^8$)					
Control	3·6±1·1	3·3±0·9	3·0±0·6	2·1±0·5	2·6±0·6
FK-506	—	3·0±1·7	1·4±0·6*†	3·9±1·8	2·2±0·8
Thymus ($\times 10^8$)					
Control	7·3±1·3	3·7±3·0	6·7±3·2	7·5±4·8	10·6±5·5
FK-506	—	4·5±2·7	4·8±1·0	9·6±1·1	7·8±2·5

Results are means ± 1 s.d. obtained from groups of six rats.

FK-506 (1 mg/kg) or drug vehicle was administered i.m. on days 0–6, inclusive.

* $P<0·01$ compared with vehicle-treated controls at the same time-point.

† $P<0·01$ compared with untreated controls on day 0.

to drug withdrawal (days 7, 14 and 21). The rise in mean serum antibody titre observed in control animals between days 4 and 21 was not apparent in FK-506-treated rats.

Mononuclear cell numbers in blood, spleen and thymus

No significant effects of FK-506 on the absolute numbers of blood or spleen mononuclear cells (MNC) were observed on day 4 compared with drug-vehicle-treated controls (Table 2). By day 7, however, there were significant reductions in blood (24%) and spleen cell numbers (53%) in the drug-treated group. Following drug withdrawal, blood and spleen cell numbers were similar to control values. In both groups, there was maximal elevation in blood MNC on day 14, followed by a reduction to pretreatment levels on day 21. Throughout the study, there were no significant differences in total numbers of thymocytes between the two groups, despite histological evidence of striking thymic medullary atrophy in animals given FK-506, which was reversed after cessation of FK-506 administration.

Analysis of blood lymphocytes

The incidence of various blood lymphocyte subpopulations, determined by flow cytometric analysis in both FK-506 and control groups at various times after immunization are shown in Fig. 1. In control animals, there was a striking (three-fold) increase in OX-12$^+$ (B) cells between days 0 and 7, which was followed by a decrease towards normal values. These changes were reflected in corresponding alterations in the relative numbers of OX-19$^+$ (*pan*-T) lymphocytes. In comparison, animals receiving FK-506 showed significantly less pronounced increases in OX-12$^+$ cells between days 0 and 7. Compared with controls, however, B cell numbers in FK-506-treated rats remained elevated above normal values following drug withdrawal.

As with OX-19$^+$ (CD3$^+$) cells, the relative numbers of W3/25$^+$ (CD4$^+$) (in particular) and OX-8$^+$ (CD8$^+$) cells were reduced following immunization; these effects were less pronounced with FK-506 treatment. There was no significant change in the peripheral blood W3/25$^+$:OX-8$^+$ (CD4$^+$:CD8$^+$) cell ratio, throughout the experimental period, in either FK-506-treated or control animals (Fig. 2).

Figure 2 also shows the very similar quantitative and sequential changes in OX-6$^+$ (MHC class II positive) cells compared with B lymphocytes that were recorded following immunization and in response to FK-506 administration. Both a reduction and a delay in the response were observed in the drug-treated group. The significant increases in proportions of OX-40$^+$ (activated CD4$^+$) and OX-39$^+$ (IL-2R$^+$) cells seen in the control group between days 0 and 7 were not observed in FK-506-treated rats until after drug withdrawal.

Analysis of spleen lymphocytes

Similar sequential changes in lymphocyte subpopulations and activation marker expression following immunization were observed in the spleens, although the differences between vehicle control and FK-506-treated groups were less pronounced than in blood (data not shown). By day 7, however, OX-8$^+$ cells were significantly higher in the FK-506 group compared with the controls, although the difference was small and the corresponding reduction in the CD4$^+$:CD8$^+$ ratio was not significant. The expression of OX-39 and OX-40 were also depressed by FK-506. As with blood, no significant differences were observed between the groups after day 7, except for a higher incidence of OX-12$^+$ cells in the drug-treated group on day 14.

Analysis of thymocytes

Accompanying the striking medullary atrophy evident in histological sections of thymuses in the FK-506 treated group by day 7, there were significant increases in the incidences of W3/25$^+$ (CD4$^+$) and OX-8$^+$ (CD8$^+$) thymocytes when compared with drug vehicle-treated controls. Accompanying this change (Table 3), were reductions, compared with vehicle-treated controls, in the incidences of OX-44$^+$ cells (mature medullary thymocytes) on days 7 and 14, and of OX-18$^+$ (MHC class I) cells on day 7. The increase in OX-6$^+$ (MHC class II) cells following immunization was unaffected by FK-506. All markers were restored to normal values by day 21 (data not shown).

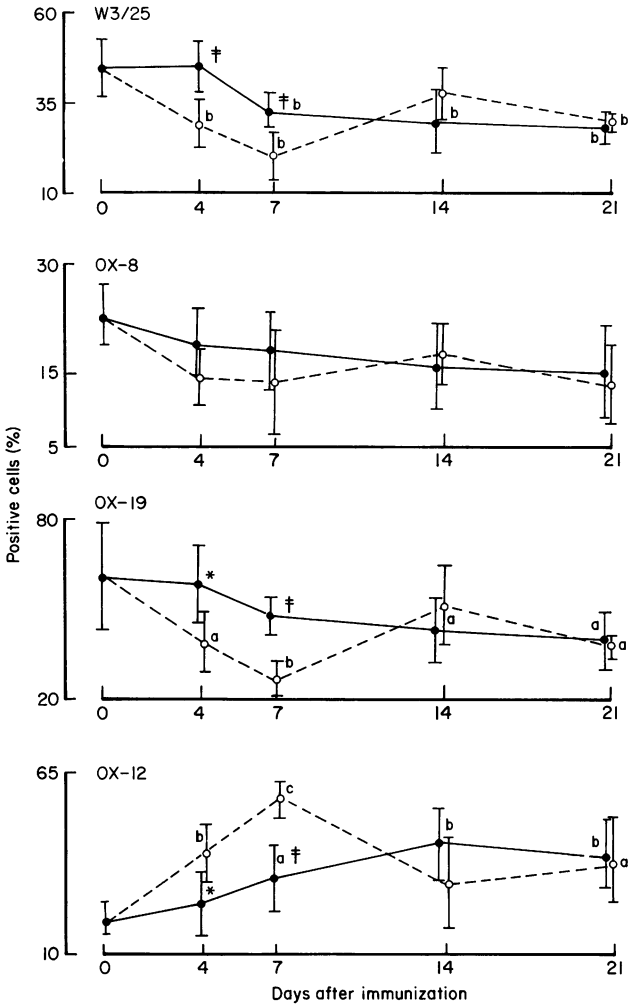

Fig. 1. Incidence of W3/25$^+$, OX-8$^+$, OX-19$^+$ and OX-12$^+$ mononuclear cells in blood of rats immunized with sheep red blood cells and treated with FK-506 (●) or drug vehicle (○) on days 0–6. Results are means ±1 s.d. obtained from groups of six rats. *$P<0.05$; ‡$P<0.01$ compared with vehicle-treated controls; (a), $P<0.05$; (b), $P<0.01$ and (c) $P<0.001$ compared with normal values.

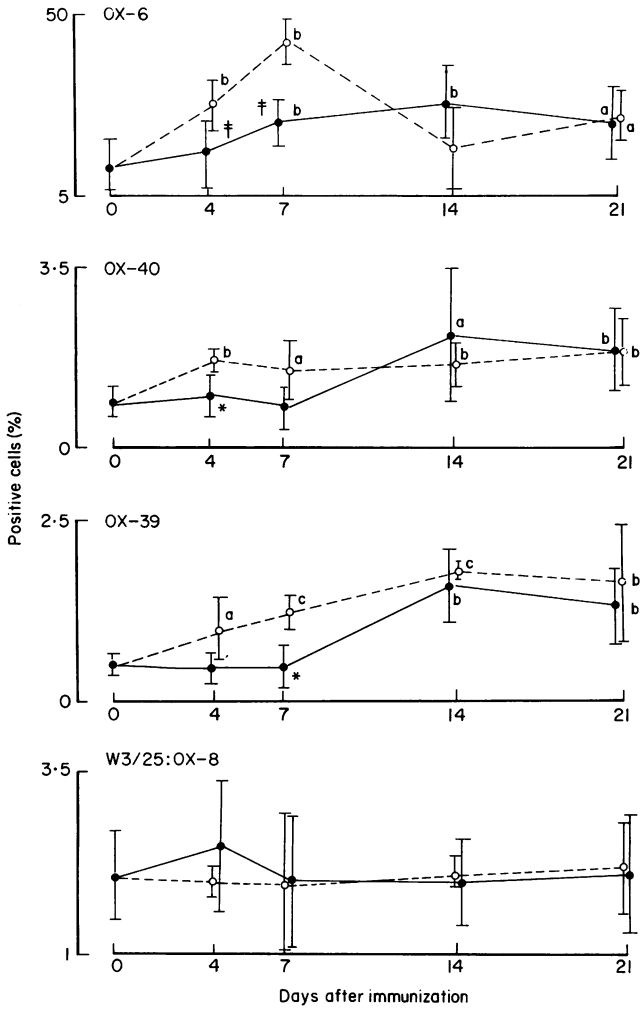

Fig. 2. Incidence of OX-6$^+$, OX-40$^+$ and OX-39$^+$ mononuclear cells and the ratio of W3/25$^+$:OX-8$^+$ cells in blood of rats immunized with SRBC and treated with FK-506 (●) or drug vehicle (○) on days 0–6. Results are means ±1 s.d. obtained from groups of six rats. †$P<0.05$; *$P<0.01$; ‡$P<0.001$ compared with vehicle-treated controls; (a) $P<0.05$; (b) $P<0.01$; and (c) $P<0.001$ compared with normal values.

DISCUSSION

Our data show that administration of 1 mg/kg FK-506 to rats for 7 days from the time of immunization with the T-dependent antigen SRBC, causes profound and sustained suppression of humoral immunity. This effect was accompanied by a transient reduction in numbers of blood or spleen lymphocytes on day 7, compared with immunized controls. During the period of drug administration, immune suppression was accompanied by significant changes, compared with immunized, vehicle-treated controls, in the relative proportions of T and B lymphocyte populations and in the expression of lymphocyte activation antigens. Our sequential analysis also revealed concomitant but reversible interference by FK-506 with thymocyte maturation.

In previous comparative studies, the immunosuppressive efficacy of FK-506 in experimental animals has been shown to be considerably greater (on a dose-equivalent basis) than that of CyA with respect to the suppression of antibody production (Kino *et al.*, 1987a), delayed-type hypersensitivity and graft-*versus*-host reactions (Kino *et al.*, 1987a), allograft rejection (Ochiai *et al.*, 1987b; Inamura *et al.*, 1988b) and autoimmune reactivity (Inamura *et al.*, 1988a; Takagishi *et al.*, 1989). There is evidence from cell culture studies that, as with CyA, this immunosuppressive activity may be mediated by inhibition of production by T cells of IL-2 and interferon-gamma (IFN-γ), although the production of B cell stimulating factor-2 (IL-6) by human lymphocytes appears to be unaffected by FK-506 (Yoshimura *et al.*, 1989b). This reported, selective inhibitory action of FK-506 on cytokine production by CD4$^+$ T cells *in vitro* may, at least in part, underlie the striking immunosuppressive activity observed in the present study, although the influence, if any, of the drug on the function of antigen-presenting cells has yet to be determined. This latter question is of particular interest, in view of considerable evidence that CyA can interfere with the antigen presenting function of rodent or human mononuclear phagocytes (Manca *et al.*, 1988; Whisler *et al.*, 1985; Varey, Champion & Cooke, 1986; Paley *et al.*, 1986; Snyder, Wright & Ting, 1987).

Table 3. Influence of FK-506 on thymocytes

Treatment	Day	Incidence (%)					
		OX-7	W3/25	OX-8	OX-44	OX-18	OX-6
Normal	—	89·1±7·5	79·3±3·5	75·8±6·7	2·2±0·1	2·6±0·7	0·9±0·3
Control	7	88·3±6·5	83·3±6·6	80·2±11·1	2·7±0·9	8·2±3·0*	4·7±2·0†
FK-506	7	89·8±8·4	89·4±5·8	92·7±5·6‡†	2·1±1·4	2·4±0·9§	4·4±2·6¶
Control	14	71·4±7·7*	84·5±7·7	83·7±8·0	5·6±1·6	4·3±1·1¶	3·7±0·2†
FK-506	14	81·3±21·8	86·2±7·4	79·4±9·4	2·2±0·7§	3·3±0·6	4·5±2·3*

Results are means ±1s.d. obtained from groups of six rats.
FK-506 (1 mg/kg) or drug vehicle was administered i.m. on days 0–6, inclusive.
* $P<0.01$; † $P<0.005$ compared with age- and sex-matched controls.
‡ $P<0.02$; § $P<0.01$ compared with vehicle-treated controls.
¶ $P<0.025$ compared with age- and sex-matched controls.

In the present study, the attenuated increase in incidence of MHC class II-positive cells seen in blood and spleen following immunization of FK-506-treated rats compared with controls was attributable to inhibition of B cell proliferation. In addition, however, FK-506 prevented the concomitant increases in activated $CD4^+$ T cells and exhibited a quantitatively similar inhibitory action on the incidence of $IL\text{-}2R^+$ cells, particularly in blood. Early results from Kino *et al.* (1987b) and Sawada *et al.* (1987) have suggested that FK-506 inhibits IL-2R expression *in vitro*, and this finding has been substantiated more recently by Yoshimura *et al.* (1989a), who also found an inhibitory effect of the drug on transferrin receptor expression. As with CyA, however, effects of FK-506 on IL-2R expression may prove difficult to interpret, since it is recognized that IL-2 up-regulates the expression of its own receptor (Smith & Cantrell, 1985; Harel-Bellan *et al.*, 1986).

The biological significance of the higher incidence of $OX\text{-}8^+$ cells in the spleens of FK-506-treated rats on day 7 compared with immunized controls is uncertain. Although statistically significant, this change was minor. Previous reports have suggested that in cardiac allografted rats, the induction of tolerance by short courses of FK-506 may be linked to sparing of donor-specific splenic suppressor cells, which can be adoptively transferred to naive recipients (Ochiai *et al.*, 1987b). Nevertheless, Yoshimura *et al.* (1989b) have recently found that FK-506 does not permit generation of human alloantigen-activated suppressor cells *in vitro*.

Our analysis of thymuses from FK-506-treated rats revealed significant and reversible reductions in the incidence of mature medullary thymocytes. This is in keeping with previous observations of thymic medullary atrophy following FK-506 administration (Stephen *et al.*, 1989) and with similar findings in CyA-treated rats (Beschorner *et al.*, 1987). FK-506 may directly destroy 'mature' thymocytes, or block the maturation of 'immature' thymocytes by suppressing the production of certain lymphokines. Further studies are needed to ascertain the influence of FK-506 on levels of thymic hormones, the expression of MHC gene products on epithelial cells and on self-MHC recognition by maturing thymocytes.

It is clear that FK-506 is an extremely potent and potentially useful immunosuppressive agent. Despite early reports of the inability of dogs to tolerate the drug (Todo *et al.*, 1987; Thiru *et al.*, 1987), recent findings indicate that it is comparatively safe in rats (Nalesnik *et al.*, 1987; Stephen *et al.*, 1989), monkeys and baboons (Todo *et al.*, 1988). The outcome of the recently commenced first preliminary investigations of FK-506 in human organ transplantation (T.E. Starzl, personal communication) is awaited with interest.

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