The effect of cyclosporin A, FK506, and rapamycin on the murine chronic graft-*versus*-host response—an *in vivo* model of Th2-like activity

R. V. BUNDICK, R. I. CRAGGS & E. HOLNESS *Department of Biochemistry, Fisons plc—Pharmaceutical Division, Research and Development Laboratories, Loughborough, UK*

SUMMARY

We have evaluated the effects of three potent immunosuppressive agents: cyclosporin A, FK506, and rapamycin, on a murine chronic graft-*versus*-host response (chronic GVHR). The chronic GVHR has previously been described to be a Th2-like response, and is characterized by a marked splenomegaly and hyper-IgE production in the early stages of the response. The effects of the immunosuppressive agents on both splenomegaly and hyper-IgE were measured 3 weeks after the induction of the chronic GVHR. Rapamycin was found to inhibit both splenomegaly and the hyper-IgE response in a dose-dependent manner. Unexpectedly cyclosporin A and FK506 were found to potentiate markedly both the splenomegaly and hyper-IgE response at low doses before exhibiting an inhibitory effect at higher doses. We propose the differences of activity seen with rapamycin compared with cyclosporin A and FK506 may be explained by their different mechanisms of action, and also by the selectivity of low dose cyclosporin A and FK506 for Th1-like lymphocytes. The implications of these observations are discussed in relation to the use of these immunosuppressives for the treatment of Th2-like diseases.

Keywords cyclosporin A   FK506   rapamycin   murine chronic GVHR   Th2-like activity

INTRODUCTION

The chronic graft-*versus*-host response (GVHR), induced in C57B1/6 × DBA/2 F_1 (B6D2F$_1$) hybrid mice by injection of non-irradiated parental DBA-2 spleen cells, has been proposed as an animal model of human lupus erythematosus [1,2]. It also has utility in the evaluation of potential immunosuppressive agents for the treatment of autoimmune diseases [3,4]. The chronic GVHR is characterized by splenomegaly; immune complex deposition in the kidney; B cell hyper-activity with hypergammaglobulinaemia; glomerulonephritis with associated proteinuria leading to kidney failure; and production of autoantibodies to nuclear antigens, dsDNA, thymic and erythrocyte antigens. The response has similar features to the spontaneous murine lupus disease seen in female NZB/NZW F_1 mice, but in the chronic GVHR the disease is expressed in a relatively short period of 10–14 weeks, whereas in the NZB/NZW F_1 it takes about 9 months for the disease to develop [5].

In the early stages of the chronic GVHR a marked elevation of IgE production has recently been observed which can be detected about 1 week after induction of the response, reaching a peak at 3–6 weeks and then maintaining a high level to week

12. The levels of IgE reach about 100-fold of normal expected levels [6,7]. The IgE response is IL-4-dependent and can be inhibited by dosing the mice with anti-IL-4 antiserum [7]. These observations indicate that the early events in the chronic GVHR contain similar mechanistic elements to those seen in allergic responses characterized by elevated IgE production.

The observation is further interesting in light of the discovery that T helper cells can be divided into distinct subsets, Th1 and Th2, depending upon their cytokine production [8], and that these subsets have functional effector properties [9]. It was soon established that the T helper cells required for IgE production were of the Th2 type [10–12], and an imbalance where Th2 cells predominate is associated with an allergic-like immunopathology.

From these data it has been proposed that the chronic GVHR is dependent on the preferential activation of Th2 cells. Spleen cells from mice undergoing the chronic GVHR have been found to produce high levels of IL-4, a Th2 cytokine, whereas they have a selective deficiency in IL-2 and interferon-gamma (IFN-γ), Th1 cytokines [13,14].

The chronic GVHR therefore is an *in vivo* model which has utility for evaluating potential immunosuppressive agents against not only autoimmune disease, but also against Th2-like-mediated diseases such as allergic responses associated with elevated IgE production.

In this study we have investigated the effects of cyclosporin

Correspondence: R. V. Bundick, Dept. of Biochemistry, Fisons plc—Pharmaceutical Division, Research and Development Laboratories, Loughborough, Leicestershire LE11 0RH, UK.

A (CsA), FK506 and rapamycin which are potent immuno-suppressives currently receiving considerable attention because of their novel mechanisms of activity [15]. All three agents appear to have selective inhibition of T lymphocyte activity [16,17]. Both CsA and FK506 are selective inhibitors of cellular immunity and have found clinical utility in transplantation. Rapamycin, although having similarities in structure to FK506, has a different mechanism of action and exhibits good suppressive activity against both cellular and humoral immunity [18–20].

MATERIALS AND METHODS

Mice

Inbred male DBA/2 mice ($H-2^d$) aged 6–8 weeks, and male C57B1/6 × DBA/2 F_1 (B6D2 F_1; $H-2^{b/d}$) mice aged 6–8 weeks were supplied by Harlan Olac (Bicester, UK). The mice were caged in groups of eight and were allowed food and water *ad libitum*.

Compounds

FK506, CsA and rapamycin were kind gifts from Fujisawa Pharmaceutical Co. (Osaka, Japan). The compounds were dissolved in ethanol, diluted in olive oil and administered as described below.

Induction of chronic GVHR and dosing protocols

Donor cell inoculum was prepared from DBA/2 mice based on the method of Doutrelepont *et al.* [7]. The spleens were removed and disaggregated through nylon mesh. Cells were resuspended by pipetting to give a single cell suspension and the aggregated debris was removed on settling out. The cells were washed twice by centrifugation and the final volume of cells was adjusted to 5×10^8 nucleated cells/ml. All procedures were carried out in RPMI 1640. The chronic GVHR was induced by the administration of 1×10^8 donor cells in a volume of 0·2 ml injected intravenously on two occasions 1 week apart (days 0 and 7). Each experiment contained groups of eight animals which were dosed once every day by the subcutaneous route. Concentrations of drugs are as described in the figures. Control groups received vehicle alone. The experiments were terminated on day 21 when the mice were weighed, bled by cardiac puncture and their spleens removed and weighed. The blood was allowed to clot and serum removed and stored at −20°C for analysis by ELISA for IgE levels.

Determination of splenomegaly

Spleens were removed on termination of the experiment and carefully cleaned of fatty tissue before weighing. The spleen : body weight ratio was calculated for each animal (mg of spleen/g bodyweight) and the group mean and standard deviation expressed. Per cent differences between control mice compared with treated mice were calculated according to the following formula:

$$100 \times \frac{\left\{ \begin{array}{c} (\text{s/b wt (non-treat)} - \text{s/b wt (normal)}) - \\ (\text{s/b wt (treat)} - \text{s/b wt (normal)}) \end{array} \right\}}{(\text{s/b wt (non-treat)} - \text{s/b wt (normal)})}$$

where s/b = ratio of spleen to body weight, normal = age-matched B6D2F_1, non-treat = GVHR mice not receiving drug; treat = GVHR mice receiving drug.

Determination of serum IgE levels

Immulon 1 microtitre plates (Dynatech, Billinghurst, UK) were coated with 50 μl at 5 μg ml LO-ME-3 monoclonal rat anti-mouse IgE heavy chain in PBS (Serotec, Oxford, UK) and left overnight at 4°C. The plates were washed twice with PBS and then blocked with 200 μl 1% bovine serum albumin (BSA). Plates were then washed twice and 50 μl duplicate samples of standard dilutions and unknown serum samples at appropriate dilutions in 0·1% BSA were added. SPE-7 mouse monoclonal IgE (ICN-Flow, Irvine, UK) was employed as a standard. The plates were incubated at room temperature for 2 h. After washing four times with PBS, 50 μl of biotinylated polyclonal anti-mouse IgE (The Binding Site, Birmingham, UK) at a 1 : 500 dilution in 0·1% BSA were added to each well. The plates were then incubated overnight at 4°C. This was followed by four further washes with PBS and addition of 50 μl of 1 : 1000 streptavidin : alkaline phosphatase conjugate (Serotec). After incubation for 50 min, the plates were washed as previously and 100 μl of the substrate (1 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine buffer pH 9·8) were added. They were then incubated at 37°C for 90 min and read at 405 nm on a Biotec plate reader (Winooski, VT). Mean differences of serum IgE between control mice and drug-treated mice were calculated.

RESULTS

Splenomegaly and IgE production in chronic GVHR mice

Induction of chronic GVHR in B6D2 F_1 mice by DBA/2 spleen cells resulted in both splenomegaly and increased levels of serum IgE (Table 1). After two injections of cells on day 0 and 7 the spleen/body weight ratio increased so that on day 21 the spleens were three-fold larger than those in normal control mice. Likewise the amount of IgE rose to a level more than 100-fold greater than that seen in control mice.

Splenomegaly and IgE production: effects of immunomodulatory agents

From day 0 of chronic GVHR induction animals were dosed daily with the immunosuppressive agents by the subcutaneous route. In each experiment the positive control animals (those

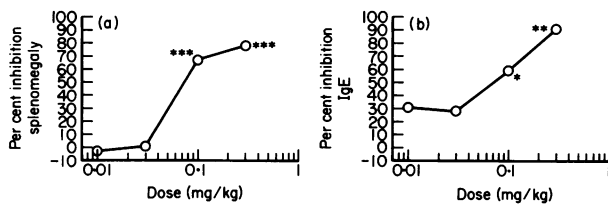**Table 1.** Splenomegaly and IgE production in the chronic graft-*versus*-host response (GVHR)

	Control: values in normal B6D2F$_1$ mice	Chronic GVHR: values at day 21 of GVHR in B6D2F$_1$ mice
Splenomegaly: ratio: mg spleen wt/g body wt ± s.d.	2·70 ± 0·35 $n = 25$	7·67 ± 1·65 $n = 28$
Total serum IgE levels: μg/ml ± s.d.	0·96 ± 1·08 $n = 8$	125·40 ± 62·70 $n = 18$

Cumulative data showing differences in splenomegaly and total serum IgE levels between control mice and 21 day chronic GVHR mice. Both comparisons were statistically significant by the Mann–Whitney U-test: $P < 0.0001$.

developing chronic GVHR) were dosed with vehicle alone. At no time was there any observation to suggest that vehicle treatment affected the progression or outcome of the chronic GVHR. Rapamycin inhibited the splenomegaly with 80% inhibition achieved at concentrations of 0·3 mg/kg (Fig. 1a) and lost activity at 0·03 mg/kg. In further experiments, rapamycin at doses >0·3 mg/kg gave inhibitions of 90% or greater (data not shown). Inhibition of IgE by rapamycin produced dose-dependent effects paralleling the observations seen in splenomegaly. Rapamycin was able to inhibit greater than 90% of the IgE production at doses of 0·3 mg/kg, and lost most of its activity at 0·03 mg/kg (Fig. 1b). At doses >0·3 mg/kg nearly maximal inhibition was observed (data not shown).

In contrast both FK506 and CsA showed a marked enhancement of splenomegaly before they had any inhibitory activity (Fig. 2a). FK506 showed maximum enhancement at 0·3 mg/kg, with spleens three-fold larger than seen in chronic GVHR mice. CsA achieved a two-fold increase in spleen size at 10 and 30 mg/kg. At concentrations of drug >1 mg/kg and 30 mg/kg, respectively, FK506 and CsA had inhibitory effects upon the splenomegaly. FK506 reached 100% inhibition at 3 mg/kg, a drug dose only one order of magnitude greater than its maximum level of enhancement. CsA only reached 67% inhibition at 100 mg/kg. Higher doses are not readily tolerated by the mice for the 21-day period of the experiment.

FK506 and CsA both induced increases in the amounts of serum IgE, similar to their effects on splenomegaly (Fig. 2b). Maximal effects of at least four-fold increases were seen at doses of 0·3 mg/kg and 30 mg/kg, respectively. These doses are the same as their maximal effects on splenomegaly. At concentrations of 3 mg/kg and 100 mg/kg, respectively, both FK506 and CsA caused maximal inhibition of the IgE response. FK506 appeared to be able to potentiate IgE production at concentrations which had no effect on spleen enlargement (0·01–0·03 mg/kg), although only one of these values was statistically significant. At concentrations <3 mg/kg, CsA had no activity on either splenomegaly or IgE production (data not shown).

Fig. 1. Per cent inhibition of chronic graft-*versus*-host response (GVHR)-induced splenomegaly and IgE production by rapamycin. Chronic GVHR mice were dosed subcutaneously daily with rapamycin. On day 21 splenomegaly and serum IgE levels in control and drug-dosed mice were measured. The effects of rapamycin on the responses were expressed as per cent inhibition where the control levels were within the range of values shown in Table 1. Statistical significance within each experiment was determined using Kruskal–Wallis non-parametric ANOVA. Where the P value for each experiment was <0·05 this was followed by the Mann–Whitney U-test to determine statistical values for each level of inhibition. $n = 8$ mice for each dose point. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. A further experiment gave similar data.
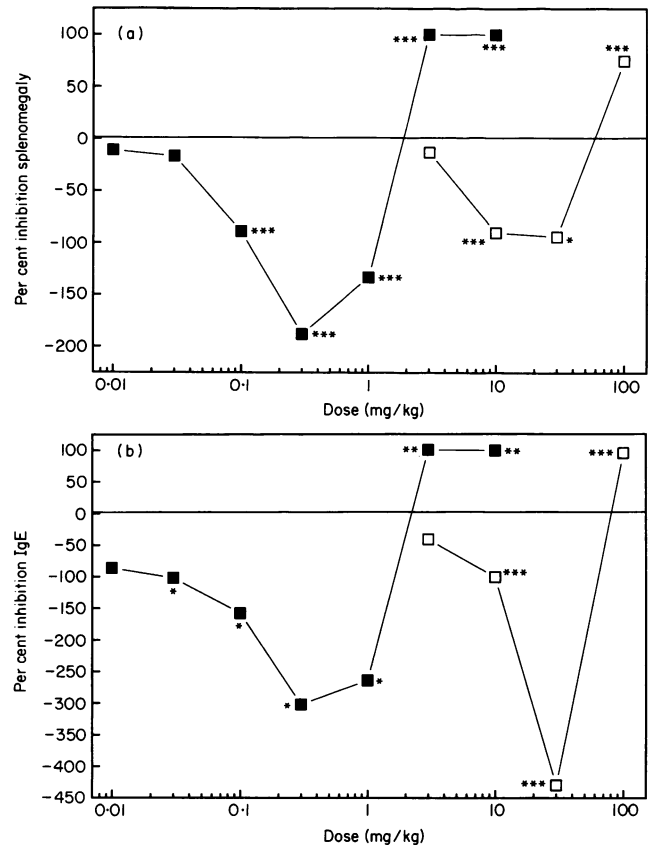
Fig. 2. Per cent inhibition of chronic graft-*versus*-host response (GVHR)-induced splenomegaly and IgE production by cyclosporin A (□) and FK506 (■). Chronic GVHR mice were dosed subcutaneously daily with cyclosporin A or FK506. On day 21 splenomegaly and serum IgE levels in control and drug-dosed mice were measured. The effects of cyclosporin A and FK506 on the responses were expressed as per cent inhibition where the control levels were within the range of values shown in Table 1. Statistical significance within each experiment was determined using Kruskal–Wallis non-parametric ANOVA. Where the P value for each experiment was <0·05 this was followed by the Mann–Whitney U-test to determine statistical values for each level of inhibition. $n = 8$ mice for each dose point. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Two further experiments gave similar data.

Comparison of immunosuppressive activity (ED$_{50}$ values)

From accumulated data, the dose of drug capable of inhibiting 50% of the control response (ED$_{50}$) values for the immunosuppressives show very close agreement between the two parameters measured. Rapamycin was the most active immunosuppressive with an ED$_{50}$ of 0·06 mg/kg and 0·05 mg/kg for inhibition of splenomegaly and IgE production, respectively. FK506 was 20-fold less active, with ED$_{50}$ values of 1·78 mg/kg and 1·59 mg/kg for splenomegaly and IgE, whilst CsA was the least potent immunosuppressive, with ED$_{50}$ values of 89·0 mg/kg and 90·0 mg/kg for splenomegaly and IgE.

DISCUSSION

This investigation shows that the immunosuppressive agent rapamycin behaves differently from CsA and FK506 in the chronic GVHR. Rapamycin mediates potent inhibition of both splenomegaly and IgE production, whereas CsA and FK506

mediate marked potentiation of both splenomegaly and IgE production at low doses, and only exhibit inhibition at higher doses. Interpretation of these results requires consideration of the known mechanisms of immunosuppressive activity elucidated for each compound, and the immune cell interactions operating in the expression of the chronic GVHR. The activity seen with rapamycin was not unexpected, as it has previously been described as having anti-proliferative activity [21,22] and an inhibitory activity on antibody production including IgE [23,24]. It is interesting that rapamycin binds to the same receptor as FK506, namely FKBP-12, which is a peptidyl prolyl *cis-trans* isomerase [25]. The complexes formed between rapamycin and FK506 with FKBP interact with different effector mechanisms inhibiting T cell proliferation [26]. Unlike CsA and FK506, rapamycin does not inhibit IL-2 production, but it is believed to interfere with cytokine activity [17]. The precise mechanism is unknown, but as a consequence of rapamycin activity a cellular p70 S6 protein kinase has been shown to be inhibited [27]. CsA and FK506, although binding to different binding proteins, have been shown to use an identical effector mechanism. Both compounds complexed with their binding protein inhibit calcineurin, a calmodulin-dependent protein phosphatase 2B [28]. This results in the inhibition of early activation gene expression responsible for the expression of cytokines such as IL-2 [29,30].

CsA and FK506 have been shown to be potent inhibitors of cell-mediated immune responses and humoral responses [31,32]. The inhibition of splenomegaly and IgE in the chronic GVHR at the higher doses was expected based on the known mechanism of activity of these compounds. The marked potentiation at the lower doses, however, was a paradoxical observation difficult to interpret in terms of mechanism of activity. Although we have yet to establish the cytokine profiles from the spleens of both treated and untreated mice, we propose that T helper cell selectivity is a possible explanation for the potentiation induced by low doses of CsA and FK506. As the chronic GVHR has been described as a Th2-like model [13], the potentiating activities of both compounds may be a result of their selective activity on the Th1 subset.

Previous published data on CsA lend support to our proposal, but as far as we are aware this is the first report of potentiation being induced by FK506. *In vitro* studies have shown CsA to have selective immunosuppressive activity at lower doses on murine Th1 clones than on Th2 clones [33]. CsA has been reported to potentiate murine IgE both *in vivo* and *in vitro* [34,35]. In murine *in vitro* studies the augmented IgE correlated to enhanced IL-4 levels and diminished production of IFN-γ, which strongly implicated a perturbance of the Th1 and Th2 cells. Furthermore, *in vivo* studies have shown CsA to increase eosinophilia and IgE/IgG1 levels in mice challenged with Aspergillus [36]. These data support the proposal that potentiation of the chronic GVHR by low dose CsA and FK506 is a consequence of both compounds exhibiting selective suppressive activity on the Th1 subset, removal of the Th1 down-regulatory effects on the Th2-driven IgE response subsequently resulting in elevated splenomegaly and IgE production. At higher doses of drug it is likely that the selectivity towards the Th1 cell is lost and there is a non-specific immunosuppression of T helper cells.

Most of the reports cited are from *in vitro* studies or disease models in the mouse. It may be that the potentiating effects of

CsA and FK506 are peculiar to the mouse, but reports from the clinic suggest that CsA may also potentiate Th2-like immunity in man [37]. We have recently shown that CsA will also potentiate IgE production *in vitro* from human peripheral blood cells (Wheeler *et al.*, in preparation). An autoimmune-like GVHR has been reported to develop in patients with solid organ allografts after the tapering of CsA dosing [38,39]. It is also interesting that CsA has proved to be more effective in those autoimmune diseases that subsequently have proved to be Th1-like than in diseases which are more Th2-like [40–43]. All these reports support CsA having selectivity towards a Th1-like response. We believe from our results that FK506 will behave similarly, and the only difference will be one of increased potency. Our data also suggest that rapamycin does not possess selectivity at the T helper cell level. The mechanism determining the selectivity of CsA and FK506 at low doses remains to be identified. Although these immunosuppressives are potent inhibitors of IL-2 and IFN-γ production, important Th1 cytokines, the Th2 cytokines IL-4 and IL-5 are equally inhibited at the single-cell level [44]. This suggests that the compounds may be acting on some component important in the mutual regulatory pathways that exist between the Th1- and Th2-like cells.

Our observations could be of importance when these immunosuppressives are employed in the clinic to treat allergic and autoimmune disorders. Although CsA, and more recently FK506, have been shown to be efficacious in a number of immunological disorders [45,46], the consequences of subsequent tapering of dosing, in an attempt to minimize the known side effects of these drugs, remain unclear. Based on epidemiological studies showing a correlation between increased serum IgE levels and asthma [47], it may be argued that a potentiation of Th2-like responses, leading to increased IgE production, will result in an exacerbation of allergic diseases and certain autoimmune disorders. However, in recent studies on the effects of prednisolone in atopic patients there was an improvement in pulmonary function despite an increase in the levels of serum IgE [48]. Interestingly, CsA administered to patients suffering from corticosteroid-dependent chronic severe asthma resulted in an improvement of the morning peak respiratory flow rate and forced expiratory volume, but the effects on IgE levels were not reported [49]. In the study CsA was administered at 5 mg/kg, which has previously been shown to be a standard immunosuppressive dose [50].

We are further investigating the proposed selectivity of these immunosuppressive agents against the T helper subsets. Confirmation of such selectivity will assist in understanding the role of these subsets in the immunopathology of immune disorders and may allow optimization of immunosuppressive therapeutic procedures.

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