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Effects of combined administration of FK 506 and the purine biosynthesis inhibitors mizoribine or mycophenolic acid on lymphocyte DNA synthesis and T cell activation molecule expression in human mixed lymphocyte cultures

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Abstract: Our objective was to obtain new information on the *in vitro* antilymphocytic action of the cytokine synthesis inhibitor FK 506 and the purine biosynthesis inhibitors mycophenolic acid (MPA; the active moiety of RS61443) and mizoribine (MZB) when used alone or in combination. When added at the initiation of six-day human mixed lymphocyte cultures (MLC), FK 506, MPA or MZB exhibited dose-dependent inhibition of T-lymphocyte DNA synthesis. FK 506, however, was 100-fold more potent than MPA, and 10000-fold more potent than MZB. Combination of FK 506 with either MPA or MZB, each at suboptional concentrations, produced no more than additive inhibitory effects on ^{3}H thymidine incorporation. Two-colour flow cytometric analysis of lymphocytes revealed that none of the drugs affected cell surface activation molecule expression (CD25 = IL-2R 55 kD α-chain, HLA-DR or CD71 = transferrin receptor [TR]) on allostimulated CD4$^+$ or CD8$^+$ cells harvested at three days of culture. By day six, however, all three agents, at levels which markedly inhibited proliferation, suppressed the expression of activation markers on both CD4$^+$ and CD8$^+$ cells. Also at day six, inhibition of activation molecule expression on CD4$^+$ cells was achieved with the combination of FK 506 and either MPA or MZB at concentrations which, on their own, were ineffective. These data provide new, additional information on the *in vitro* antilymphocytic action of FK 506, MPA and MZB when used alone and in combination.

Introduction

The efficacy and clinical potential of a variety of new immunosuppressive drugs when used alone or in combination are currently the subjects of critical evaluation.[1,2] The macrolide antibiotic FK 506 exhibits a similar molecular action to cyclosporin A (CsA)[3] and interferes with T cell proliferation stimulated via the T cell receptor (TCR)/CD3 pathway. This effect is achieved by blocking of the transcription of genes encoding interleukin-2 (IL-2) and other cytokines.[4] FK 506 has recently been shown to be effective in the prevention and rescue of human organ allograft rejection and may have a superior therapeutic index to CsA both in transplantation[5–8] and autoimmune disease.[9]

Mycophenolic acid (MPA; the active moiety of RS-61443 = mycophenolate mofetil) and mizoribine (MZR) are strong inhibitors of enzymes which catalyse the *de novo* synthesis of guanine nucleotides that are essential for T cell replication. They thus act later in the cell cycle than FK 506. By decreasing nucleotide availability, however, MPA and MZR may also inhibit signal transduction and the synthesis of cell surface proteins and receptors.[10–15] Both drugs have recently been shown to inhibit T cell responses *in vitro*.[16–18] In addition, MPA or MZR suppresses transplant rejection and each drug has been reported to act synergistically with CsA to prolong survival of experimental organ allografts.[19–23] Moreover, MPA and MZR appear to be less toxic than azathioprine to the intestine and bone marrow. Thus, both MPA and MZR

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are of prospective clinical value as adjunctive immunosuppressive agents for use in combination with FK 506.

Objectives

To examine further the effects of FK 506, MPA and MZR on human T cell activation, we investigated the expression of several activation molecules (CD25 = IL-2R 55 kD α-chain, HLA-DR and CD71 = transferrin receptor[TR]) on allostimulated $CD4^+$ and $CD8^+$ lymphocytes cultured in the presence of FK 506, MPA or MZR or when treated with combinations of FK 506 and either purine nucleotide synthesis inhibitor. It was anticipated that the results obtained would provide additional, new information on the effect of these drugs on lymphocyte activation and assist in the design of drug combination therapies.

Materials and methods

FK 506 (Fujisawa Pharmaceutical Co, Osaka, Japan) and MPA (Sigma Chemical Co, St Louis, MO, USA) were dissolved initially in absolute ethanol. MZB (Asahi Chemical Industry Co Ltd, Tokyo, Japan) was dissolved in sterile, distilled water before further dilution in cell culture medium. Peripheral blood mononuclear cells were isolated over Ficoll-Isopaque from heparinized samples obtained from healthy adult volunteers of both sexes. One-way mixed lymphocyte reaction (MLR) cultures were established in RPMI 1640, supplemented with 25 mmol/L Hepes buffer and 100 U/ml gentamicin, with 10% normal human AB serum. Equal numbers (5×10^4 well) of responder and irradiated (2000 Rad) stimulator cells were set up in flat-bottomed microtitre plates in a total volume of 0.2 ml. Cells were cultured for three or six days. [^{3}H]-thymidine (1 μCi/well) was added for the final 16 hours of culture. The cells were harvested onto glass fibre discs, using a multiple harvester and [^{3}H]-thymidine uptake was estimated using a 1205 Betaplate liquid scintillation counter (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Results are expressed as mean counts per minute (cpm).

Cultured mononuclear cells were washed and the expression of activation antigens was detected as described previously[24] by staining cells with FITC-conjugated anti-CD25 (IL-2 receptor, α chain), anti-HLA-DR or anti-CD71 (TR) mouse IgG monoclonal antibodies and either phycoerythrin (PE)-conjugated anti-CD4 or anti-CD8 mouse monoclonal antibodies. All antibodies were from DAKO and were used at a final 1:10 dilution. FITC- and PE-conjugated mouse IgG were used as background control. After washing, cells were fixed with 1% w/v paraformaldehyde, followed by analysis in a FACSTAR flow cytometer (Becton-Dickinson, Rariton, NJ, USA). Five thousand cells were counted for each sample. Significances of differences between means were determined using Student's *t*-test.

Results

The influence of various concentrations of FK 506, MPA or MZB, added at the start of culture, on the uptake ^{3}H-TdR in six-day human MLC is shown in Figure 1. Each drug exhibited a dose-related, inhibitory effect with maximal inhibition of DNA synthesis (85–97%) being achieved with 1.2 nM FK 506, 0.3 μM MPA and 386 μM MZB. Combinations of moderately effective concentrations of FK 506 and either MPA or MZB produced no more than an additive inhibitory effect on cell proliferation.

The effects of the three immunosuppressive agents on human T cell surface IL-2R, HLA-DR and TR expression in three- and six-day MLC were examined by two-colour flow cytometric analysis. Cultures harvested after three days in the presence of FK 506, MPA or MZB showed no significant change in the incidence of $CD4^+$ or $CD8^+$ cells co-expressing any of the activation markers (data not shown). By day six, however, there were significant inhibitory effects of all three drugs on the incidence of both $CD4^+$ and $CD8^+$ cells co-expressing each of the markers (Figure 2). At concentrations of each drug which gave maximal inhibition of DNA synthesis (Figure 1), but not at tenfold lower concentrations (which were moderately antiproliferative), FK 506, MPA and MZR significantly inhibited ($p < 0.025$) expression of IL-2R on both $CD4^+$ and $CD8^+$ cells. The extent of suppression (50–60%)

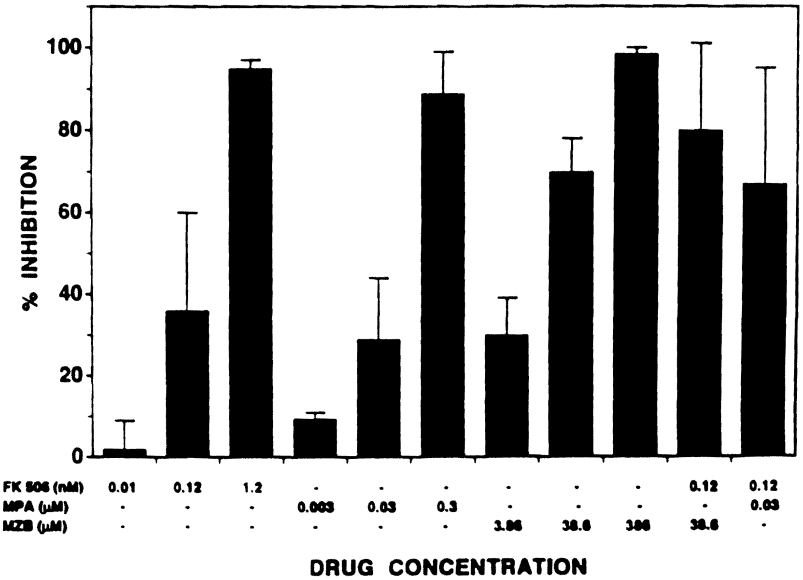

Figure 1 The influence of various concentrations of FK 506, MPA and MZB and of drug combination on human MLR. Uptake of ^{3}HTdR was quantitated over the last 16 hours of six-day cultures. Results are means ± 1 SD obtained from three separate experiments.

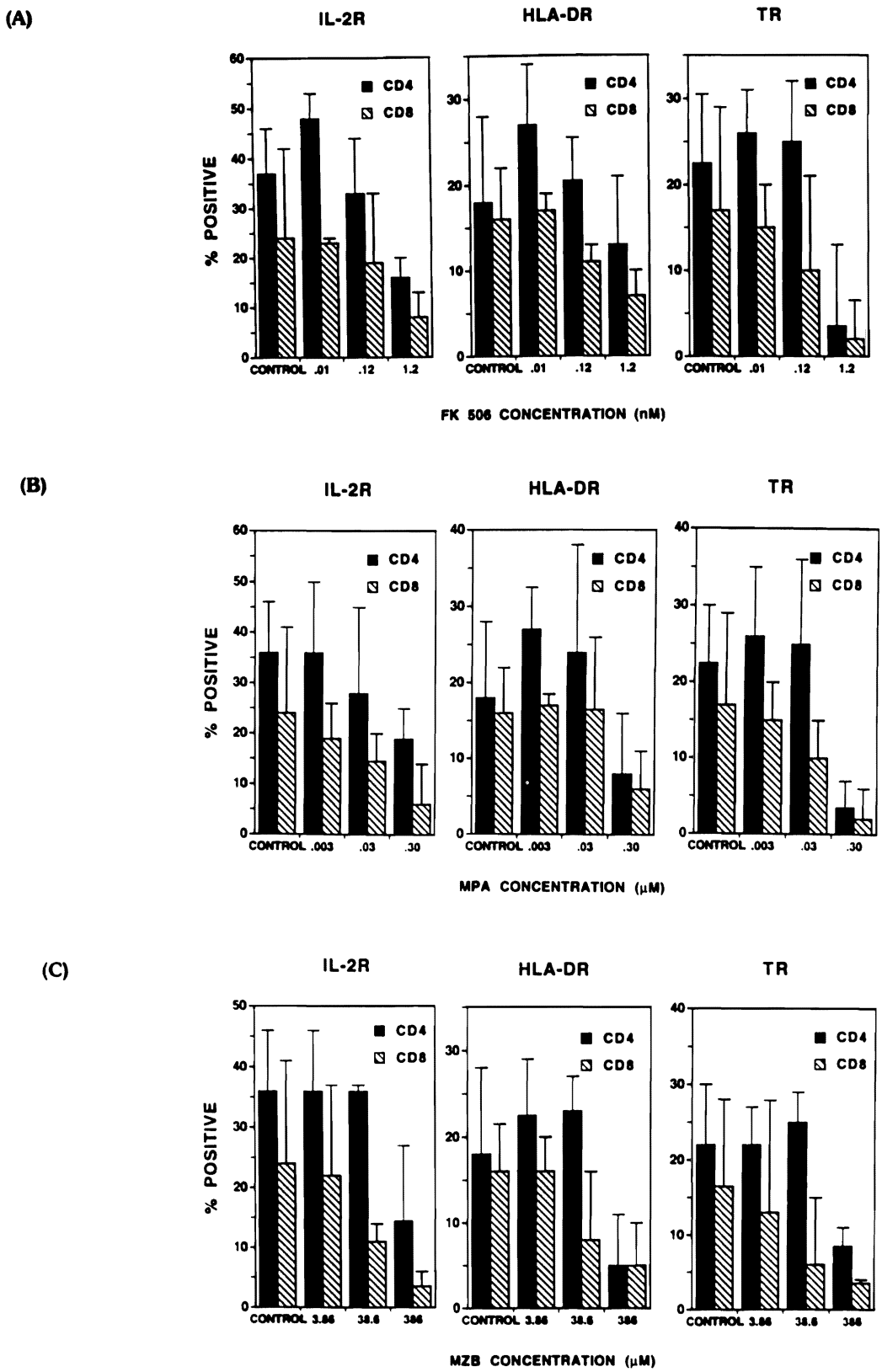

Figure 2 The incidence of $CD4^+$ and $CD8^+$ lymphocytes expressing IL-2R, HLA-DR or TR in (A) FK 506-treated; (B) MPA-treated; and (C) MZB-treated MLC determined by two-colour flow cytometric analysis six days after establishment of cultures. Results are means ± 1 SD obtained from three separate experiments.

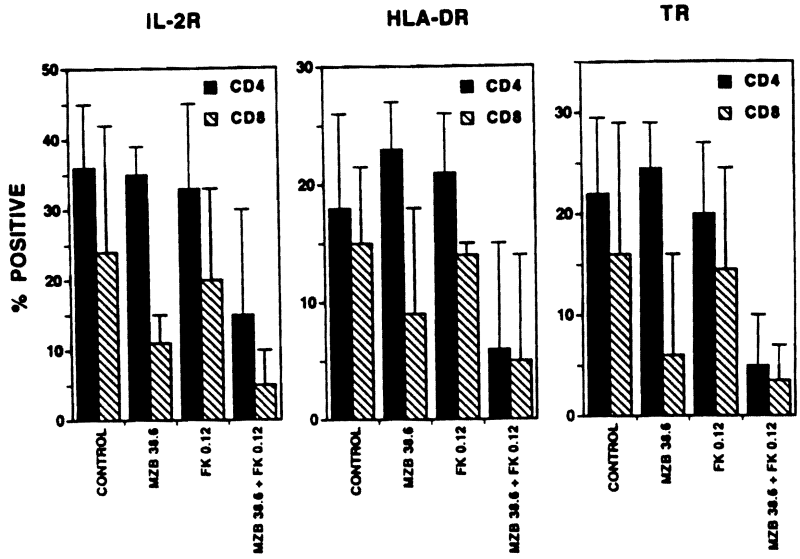

Figure 3 The incidence of $CD4^+$ and $CD8^+$ lymphocytes expressing IL-2R, HLA-DR or TR in six-day MLC cultures treated with concentrations of FK 506 and MZB which, on their own, did not affect the expression of these markers. Results are means ± 1 SD obtained from three separate experiments. MZB μM; FK 506 nM.

was similar for both T cell subsets, although MZB was especially effective in suppressing $CD8^+$ IL-2R expression (>80%). MZR was also the most effective agent and both MZR and MPA more effective than FK 506 in suppressing HLA-DR expression. Differences between the effects of these drugs, however, were not stastically significant. The incidence of $CD4^+$ and $CD8^+$ cells expressing TR was also inhibited ($p < 0.02$) by all three drugs and to a greater extent than IL-2R and HLA-DR, although as with the latter activation markers, at only the highest drug concentration tested.

Combinations of FK 506 and either MPA or MZB at concentrations which, on their own caused only moderate inhibition of DNA synthesis and no significant reduction in cell surface marker expression, reduced the incidence of IL-$2R^+$, HLA-DR^+, and TR^+ cells (Figure 3; data shown for FK 506-MZB combination only).

Discussion

In this study, we have shown that the immunosuppressive agents, FK 506, MPA (the active moiety of RS-61443) and MZB each has the capacity to profoundly inhibit T cell proliferation and the expression of T cell surface activation molecules following allostimulation. In each instance, significant suppression of IL-2R (CD25; 55 kD α-chain), HLA-DR and TR (CD71) on $CD4^+$ and $CD8^+$ cells was observed at six but not at three days of culture. Moreover, this effect was achieved at drug concentrations which caused at least 80% inhibition of cell proliferation; lower concentrations, which produced <70% inhibition of DNA synthesis, did not suppress activation marker expression.

Despite these similarities in the effects of the three agents, marked differences in potency were recorded. FK 506 proved highly effective in inhibiting cell proliferation at approximately 1 nM, whilse MPA and MZB were 100- and 10 000-fold less potent. Our data also confirm the distinct modes of action of FK 506 on the one hand and MPA and MZB on the other. FK 506 has been shown to inhibit selectively the activation and proliferation of T cells stimulated via the T cell receptor/CD3 pathway.[4,25,26] Following binding of FK 506 to its intracellular, cytosolic receptor FK 506 binding protein (FKBP; predominant member FKBP-12), the FK 506-FKBP-complex binds to the calcium-activated phosphatase calcineurin.[27] Current thinking is that FK 506 may block dephosphorylation of the cytosolic component of the nuclear factor of activated T cells (NFAT)[28] that is required for transcription of IL-2 mRNA.

Here we have extended our previous observations[24,29] that FK 506 inhibits activation molecule expression on OKT3 stimulated or alloactivated human lymphocytes. Thus, in the present study, using two-colour immunofluorescence staining, we have shown that FK 506 inhibits IL-2R, HLA-DR and TR on both $CD4^+$ and $CD8^+$ alloactivated T cells. We could not confirm an earlier report by Kino *et al.*[25] that FK 506 inhibited IL-2R (CD25) more markedly on $CD8^+$ than on alloactivated $CD4^+$ cells.

The antiproliferative effects of MPA and MZB differ distinctly from those of FK 506. Both former drugs are purine biosynthesis inhibitors. It has been reported that immunosuppressive doses of MPA do not deplete lymphocytes and may have no effect on DNA synthesis in nonlymphoid tissues, such as gut epithelium or on most bone marrow progenitor cells.[16] We have observed that neither MPA nor MZB affects Con A-induced T cell cytokine gene expression (IL-2, IL-4, IFN-γ or IL-10) (data not shown). Little previous data are available concerning the effects of MPA or MZB on cytokines. Turka *et al.*[17] however, recently reported failure of MZB to inhibit phorbol myristate acetate (PMA) + ionomycin-induced human T cell IL-2 gene expression or cell surface 55 kD IL-2R expression. In the latter instance, the antigen was studied after T cells were stimulated with PMA + ionomycin + anti-CD28, a much more powerful stimulus than the alloantigen used in the present study. This difference in strength of stimulus may explain the apparent discrepancy between the two studies.

Advances in our understanding of events underlying T cell activation have helped pinpoint sites of action of immunosuppressive drugs and aided in the design of new drug combination therapies. In this study, combination of a suboptimal

concentration of FK 506 with either MPA or MZB was found to inhibit cell proliferation at approximately the level seen with tenfold higher concentrations of FK 506. The apparent selectivity of MPA and MZB for lymphoid T cells *in vivo* suggests that use of FK 506 together with either MPA or MZB may be useful for the immunosuppressive therapy of human allograft rejection. Indeed, Sollinger *et al.*[22] reported recently on the efficacy and safety of MPA (RS-61443) in combination with low doses of CsA and prednisone in clinical renal transplantation. Furthermore, like FK 506, neither MPA nor MZB has been reported to affect the bone marrow at immunosuppressive doses,[16,17] a possible advantage over azathioprine in minimizing the toxicity of drug combination therapy.

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