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RAPAMYCIN BUT NOT FK506 INHIBITS THE PROLIFERATION OF MONONUCLEAR PHAGOCYTES INDUCED BY COLONY-STIMULATING FACTORS¹

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FK506, CsA, and rapamycin are potent inhibitors of T lymphocyte activation; relatively little is known of their effects on cells of the monocyte/macrophage lineage. Studies were undertaken to determine the effects of these drugs on the proliferative response of bone marrow-derived mononuclear phagocytes (BMMP) to CSFs. Rapamycin inhibited the proliferation of BMMP cultured in the presence of 10% L cell-conditioned medium, used as a source of macrophage CSF. The inhibition by rapamycin was dose dependent and apparent at concentrations of 0.1 nM or greater. In a similar fashion, rapamycin inhibited the proliferation of BMMP stimulated by the recombinant forms of murine IL-3 and murine granulocyte-macrophage CSF, and human macrophage CSF. In contrast, neither FK506 nor CsA at concentrations as high as 1000 nM diminished the proliferation of BMMP cultured under identical conditions. FK506, but not CsA, blocked the inhibitory effects of rapamycin on the response of BMMP to CSFs. In summary, these data indicate that rapamycin inhibits the proliferation of BMMP in response to CSFs. These results imply that patients receiving rapamycin, but not FK506 or CsA, may have an impaired ability to generate a functional mononuclear phagocyte population.

antibiotics derived from *Streptomyces tsukubaensis* (1-3) and *Streptomyces hygroscopicus* (4, 5), respectively. CsA, a cyclic peptide, is an unrelated fungal metabolite (6, 7). All 3 compounds exhibit potent immunosuppressive activity in vivo (8-11) and in vitro (12-14). Although FK506 and CsA are structurally unrelated, they exhibit similar mechanisms of action. Both inhibit the early events in T cell activation that occur subsequent to antigen-TCR interaction and that culminate in the expression of early T cell activation genes (15). FK506 is 10-100 times more potent than CsA in suppressing the proliferation of mitogen- and alloantigen-activated T lymphocytes in vitro (12, 13). In addition, FK506 is more effective than CsA in suppressing graft rejection after organ transplantation in animal models (16). Both FK506 and CsA are used clinically to prevent graft rejection in humans (17, 18).

In contrast to either FK506 or CsA, rapamycin inhibits cytokine-driven T lymphocyte proliferation rather than the activation of cells induced by the antigen-TCR interaction (19). FK506 and rapamycin bind to the same intracellular protein, FK-binding protein (FKBP),* which is distinct from the intracellular binding site of CsA, cyclophilin (20-22). As a consequence, FK506 and rapamycin act as reciprocal antagonists exerting mutually exclusive effects on T cell proliferation.

FK506 and rapamycin are structurally related, macrolide

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* Abbreviations: BMMP, bone marrow-derived mononuclear phagocyte; FBS, fetal bovine serum; FKBP, FK-binding protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; LCM, L cell-conditioned medium; M-CSF, macrophage colony-stimulating factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; rh, recombinant human; rm, recombinant murine.

eration (13). Like FK506 and CsA, rapamycin effectively suppresses allograft rejection in animal models (23).

While the effects of rapamycin, FK506, and CsA on T lymphocytes are well documented, less is known of the effects of these drugs on other immune cell types, including mononuclear phagocytes. The proliferation and differentiation of bone marrow-derived mononuclear phagocytes (BMMP) from committed myeloid progenitors represent important features of the host's immune system (24). The growth factors responsible for the generation of a functional mononuclear phagocyte population constitute a family of glycoproteins termed the colony-stimulating factors. These factors include macrophage CSF (M-CSF), which stimulates the maximum proliferation of cells in this lineage, as well as granulocyte-macrophage CSF (GM-CSF) and IL-3. These CSFs may act alone or in combination to promote the proliferation and survival of BMMP *in vivo* and *in vitro* (24).

The present study was undertaken to investigate the effects of rapamycin, FK506, and CsA on the ability of BMMP to proliferate in response to CSFs. Here we report that CSF-driven BMMP proliferation is inhibited by pharmacological concentrations of rapamycin, but not of FK506 or CsA. These findings constitute one of the first reports to demonstrate that rapamycin differs from both FK506 and CsA in its effect on cells of the myeloid lineage. These data may have important implications with regard to the clinical use of rapamycin, FK506, and CsA in patients requiring immunosuppressive therapy, and to the mechanisms that underlie growth factor signaling in mononuclear phagocytes.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old female C57BL/6J mice purchased from Jackson Laboratories, Bar Harbor, ME, were used in all experiments. Animals were housed in accordance with the guidelines proposed by the Institute of Laboratory Animals Resources, National Research Council.

BMMP. BMMP were obtained as described previously (25, 26). Briefly, bone marrow was harvested from the femurs of mice killed by cervical dislocation. The cells were suspended in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sterile Systems, Inc., Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 20% heat-inactivated equine serum (Sterile Systems) and 20% serum-free L 929 cell-conditioned medium (LCM), which served as a source of M-CSF. Bone marrow cells (2×10^5) in 20 ml of medium were seeded into 10-cm bacteriological grade polystyrene petri dishes, and the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. On day 7 an additional 2 ml of LCM were added to all cultures. The cells were harvested after 11 or 12 days incubation, at which time they constituted a pure, relatively immature population of BMMP (25, 26).

CSFs. Recombinant human (rh) M-CSF and recombinant murine (rm) GM-CSF were the generous gifts of Immunex Corp., Seattle, WA; rmIL-3 was purchased from Genzyme Corp., Boston, MA. In preliminary experiments, 10,000 U/ml rhM-CSF, 1,000 U/ml rmGM-CSF, and 100 U/ml rmIL-3 stimulated the maximum proliferation of BMMP cultured under the conditions described.

Immunosuppressants. Rapamycin and FK506 were gifts obtained from Wyeth-Ayerst Laboratories, Princeton, NJ, and from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, respectively. CsA was obtained from Sandoz Pharmaceuticals, Basel, Switzerland. Stock 10-mM drug concentrations were prepared in absolute ethanol. Dilutions were made in RPMI 1640 medium supplemented with 10% FBS and antibiotics immediately before experimental use.

Assay of the effect of CSFs and drugs on the proliferation of BMMP.

Microtiter plates were inoculated with BMMP suspended in RPMI medium supplemented with 10% FBS and antibiotics (5×10^3 cells/well) and the plates were incubated for 2 hr to allow the cells to attach. FK506, rapamycin, or CsA (ranging in concentration from 0.1 nM to 1000 nM) was then added with or without LCM, rhM-CSF, rmGM-CSF, or rmIL-3 and cells were cultured for an additional period ranging from 4 hr to 7 days.

Assessment of cell proliferation. The proliferation of BMMP was assessed by one of the following 3 methods. (a) Metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): The ability of the BMMP to metabolize the tetrazolium salt MTT was used as a measure of cell proliferation (27). The cells were incubated with 5 mg/ml MTT (Sigma Chemical Co., St. Louis, MO) during the last 4 hr of the culture period. To solubilize the formazan product of MTT metabolism, an equal volume of 10% SDS in 0.1 N HCl was added to each well and the plates were incubated for an additional 18 hr at 37°C. The plates were read on a multiwell scanning spectrophotometer (ELISA reader) using a 570-nm test filter and a 630-nm reference filter.

(b) [³H]Thymidine incorporation: The cells were pulsed with 1 µCi/well [methyl-³H]thymidine (ICN, Costa Mesa, CA) during the last 18 hr of the culture period. The cells were collected using an automated cell harvester and [³H]thymidine incorporated into DNA was determined by liquid scintillation counting.

(c) Quantitation of cell number: The number of cells per well was assessed by lysing the cells with a 3% solution of hexadecyltrimethylammonium bromide (Fisher Scientific Co., Fair Lawn, NJ) and 1 mM EDTA in 0.85% saline, and counting the nuclei microscopically using a hemocytometer (28, 29).

Con A-induced splenocyte proliferation. Freshly isolated mouse splenocytes were suspended in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 1 mM L-glutamine, 1% essential and nonessential amino acids, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were seeded into microtiter plates (1×10^5 cells/well) and incubated 3 days in the presence or absence of 1 µg/ml Con A and FK506, rapamycin, or CsA ranging in concentration from 0.1 to 1000 nM. [³H]Thymidine incorporation into DNA during the last 18 hr of the culture period was used as a measure of cell proliferation.

Statistical analysis. Means were compared using a nonpaired Student's *t* test; *P* < 0.05 was statistically significant.

RESULTS

Rapamycin inhibits the proliferative response of BMMP to 10% LCM. Studies were performed to examine the effects of rapamycin, CsA, and FK506 on the kinetics of BMMP proliferation in response to 10% LCM, used as a source of M-CSF. Proliferation was assessed in terms of the ability of cells to metabolize the tetrazolium salt MTT. As shown in Figure 1, the inhibitory effect of 10 nM rapamycin on cell proliferation was evident on days 3, 5, and 7 of a 7-day incubation period. In contrast, neither FK506 nor CsA exerted an adverse effect on the proliferation of BMMP during this time. It is pertinent to note that none of the 3 immunosuppressants tested affected the degeneration or death of cells characteristic of BMMP cultured in the absence of an exogenous source of CSFs. Over the course of a 3-day incubation period, BMMP cultured in the absence of added CSFs exhibited a 5- to 10-fold loss in capacity to metabolize MMT regardless of whether FK506, CsA, or rapamycin was present at concentrations ranging from 0.001 to 1000 nM (data not shown).

Rapamycin inhibits the proliferation of BMMP in a dose-dependent fashion. Rapamycin, CsA, and FK506 were tested over a broad range of concentrations for their effects on the proliferative response of BMMP to 10% LCM. These effects

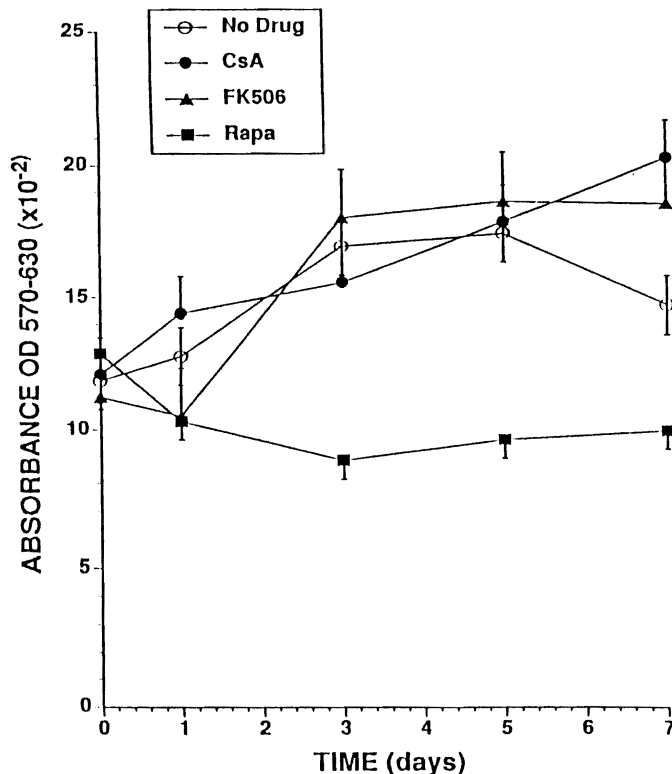


FIGURE 1. Growth kinetics of BMMP cultured in the presence of CsA, FK506, or rapamycin. BMMP (5×10^3 /6-mm well) were cultured in the presence of 10% LCM with or without 10 nM CsA, FK506, or rapamycin. Cell proliferation was assessed by the addition of MTT during the last 4 hr of the culture period. Values are the means \pm SD OD₅₇₀₋₆₃₀ derived from 4 identical wells in 1 experiment representative of 5 similar experiments. Cell growth in cultures that contained rapamycin was significantly less on days 3, 5, and 7 ($P < 0.001$).

were analyzed on the fifth day of incubation when the proliferation of cells peaked in control, untreated cultures. Rapamycin inhibited BMMP proliferation in response to LCM in a dose-dependent fashion (Fig. 2). Significant inhibitory effects were noted with 0.1 nM rapamycin; rapamycin at 10 nM or greater inhibited cell proliferation maximally. Neither FK506 nor CsA at concentrations ranging from 0.1 to 1000 nM affected the proliferation of cells cultured in the presence of 10% LCM.

Cell proliferation assessed in terms of MTT metabolism correlates with tritiated thymidine incorporation and cell number. To ensure that the results obtained by analysis of MTT metabolism were a true reflection of BMMP proliferation, cell number and [3 H]thymidine incorporation into DNA were used in parallel experiments to assess proliferation. The addition of 10 nM rapamycin to BMMP cultured in the presence of 10% LCM resulted in a 50% decrease in MTT metabolism relative to untreated control cultures (Table 1). Similarly, cell cultures that contained rapamycin exhibited an approximate 75% decrease in both [3 H]thymidine incorporation and cell number. Neither FK506 nor CsA had an effect on BMMP proliferation as determined by each of the 3 assays.

Rapamycin inhibits the proliferative response of BMMP to the recombinant forms of human M-CSF, murine GM-CSF, and IL-3. The effects of rapamycin, CsA, and FK506 on the

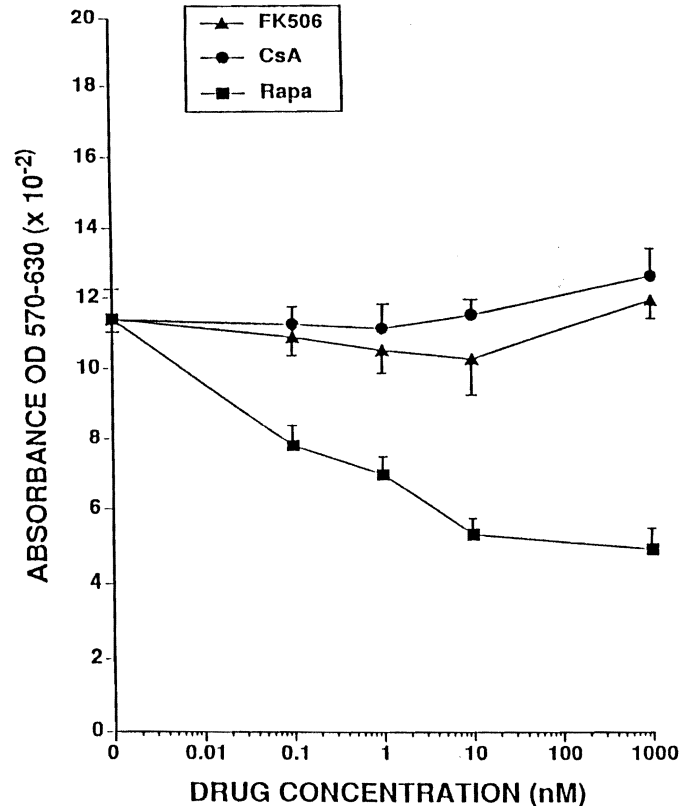


FIGURE 2. Rapamycin inhibits BMMP proliferation in a dose-dependent fashion. BMMP (5×10^3 /6-mm well) were incubated in the presence of 10% LCM and increasing concentrations of CsA, rapamycin, or FK506. On the fifth day of culture, MTT metabolism was used to assess cell proliferation. Data are the means \pm SD OD₅₇₀₋₆₃₀ readings obtained from 4 identical wells in 1 experiment. Three similar experiments gave comparable results. Cells treated with rapamycin were significantly less than untreated control cells at all concentrations tested ($P < 0.001$).

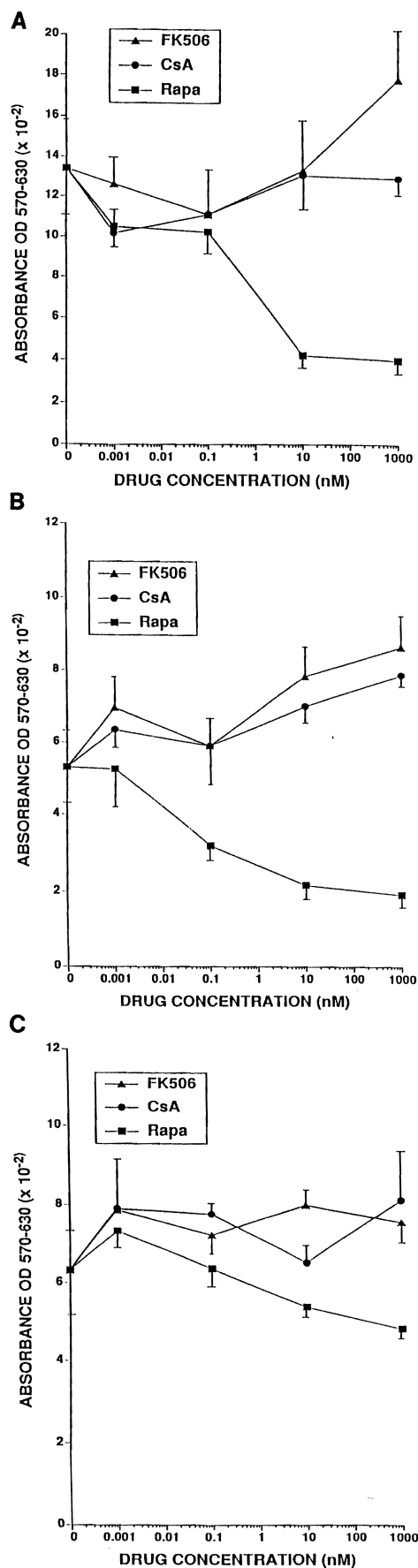
TABLE 1. Rapamycin inhibits the proliferation of BMMP: correlation between MTT metabolism, [3 H]thymidine incorporation, and cell number^a

Drug	MTT Metabolism (OD ₅₇₀₋₆₃₀ $\times 10^2$)	[3 H]Thymidine incorporation (cpm)	Cell number/well ($\times 10^{-3}$)
Control	13.9 \pm 0.35	13,125 \pm 1,650	13.2 \pm 3.3
Rapamycin	7.2 \pm 0.14 ^b	3,210 \pm 890 ^b	3.0 \pm 0.8 ^b
FK506	15.0 \pm 0.25	10,798 \pm 4,873	12.1 \pm 2.6
CsA	14.8 \pm 0.59	14,827 \pm 1,922	11.6 \pm 2.7

^a BMMP (5×10^3 cells/6-mm well) were cultured in the presence of 10% LCM with or without rapamycin, FK506, or CsA at 10 nM concentrations. All assessments of proliferation were performed on day 5. Data are the means \pm SD of 4 identical wells obtained in a single experiment representative of 3 similar experiments.

^b Significantly less than the comparable control value, $P < 0.001$.

proliferative response of BMMP to recombinant CSFs correlated with their effects on the response of cells to LCM. Rapamycin at a concentration equal to or greater than 10 nM inhibited the proliferation of BMMP cultured in the presence of rhM-CSF (Fig. 3A); rapamycin (≥ 0.1 nM) inhibited the proliferation of cells induced by rmIL-3 (Fig. 3B). In addition, 1000 nM rapamycin inhibited the proliferation of cells cul-



tured in the presence of rmGM-CSF (Fig. 3C). In contrast, neither FK506 nor CsA at concentrations ranging from 0.1 to 1000 nM adversely affected the proliferative response of cells to rhM-CSF, rmGM-CSF, or rmIL-3. In fact, the addition of 1000 nM FK506 appeared to increase the growth of BMMP cultured in the presence of rhM-CSF and rmIL-3. CsA at 1000 nM had a similar effect on the growth of cells cultured in the presence of rmIL-3.

Rapamycin, FK506, and CsA inhibit the proliferative response of splenocytes to Con A. Experiments were performed to compare the effects of rapamycin, FK506, and CsA on mitogen-stimulated splenocyte proliferation to their effects on BMMP proliferation. Normal mouse splenocytes were cultured in the presence of 1 μ g/ml Con A and increasing concentrations of drug. Splenocytes cultured with either FK506 or rapamycin at concentrations equal to or greater than 0.1 nM and 0.001 nM, respectively, exhibited a marked reduction in cell proliferation relative to cells cultured in the absence of drugs (Fig. 4). Significant inhibition by CsA was observed at concentrations of 10 nM and greater. Thus, FK506 and CsA at pharmacological concentrations that had no effect on the proliferation of BMMP strongly suppressed the blastogenic response of splenocytes to Con A. In contrast, rapamycin at comparable concentrations inhibited the proliferation of both BMMP and splenocytes.

FK506, but not CsA, antagonizes the inhibitory effects of rapamycin on BMMP proliferation. FK506, but not CsA, antagonizes the inhibitory effect of rapamycin on the proliferative response of T lymphocytes to selected stimuli, i.e., IL-2 and PMA (13). Experiments were undertaken, therefore, to determine whether FK506 also antagonized the effect of rapamycin on the proliferative response of BMMP to CSFs. As shown in Figure 5, rapamycin inhibited the proliferation of cells cultured in the presence of 10% LCM; FK506 and CsA had no effect. The inhibitory effect of rapamycin on BMMP proliferation was prevented by the addition of an equimolar concentration of FK506. CsA failed to prevent the inhibitory effect of rapamycin on BMMP proliferation.

DISCUSSION

Previous studies have given little attention to the effects of immunosuppressants on the response of myeloid cells to CSFs. In the present study, pharmacological concentrations of rapamycin inhibited the proliferation of murine BMMP cultured in the presence of LCM, a source of M-CSF. Inhibition was evident regardless of the method used to assess cell growth, i.e., MTT metabolism, [3 H]thymidine incorporation, or quantitation of cell number. The effect of rapamycin was dose dependent and apparent at concentrations comparable

FIGURE 3. Rapamycin inhibits the proliferative response of BMMP to rhM-CSF, rmGM-CSF, and rmIL-3. BMMP (5×10^3 cells/well) were incubated in the presence of (A) 10,000 U/ml rhM-CSF, (B) 100 U/ml rmIL-3, or (C) 1000 U/ml rmGM-CSF and increasing concentrations of FK506, rapamycin, or CsA. MTT metabolism was determined on day 5 of culture. Data are the means \pm SD $OD_{570-630}$ derived from quadruplicate wells in single experiments representative of at least 3 similar experiments. Values obtained from untreated and drug-treated cultures are significantly different: (A) $P < 0.001$ for rapamycin at 10 nM and 1000 nM; (B) $P < 0.001$ for rapamycin at 0.1 nM, 10 nM, and 1000 nM, and $P < 0.001$ for FK506 and CsA at 10 nM and 1000 nM; (C) $P < 0.02$ for rapamycin at 1000 nM.

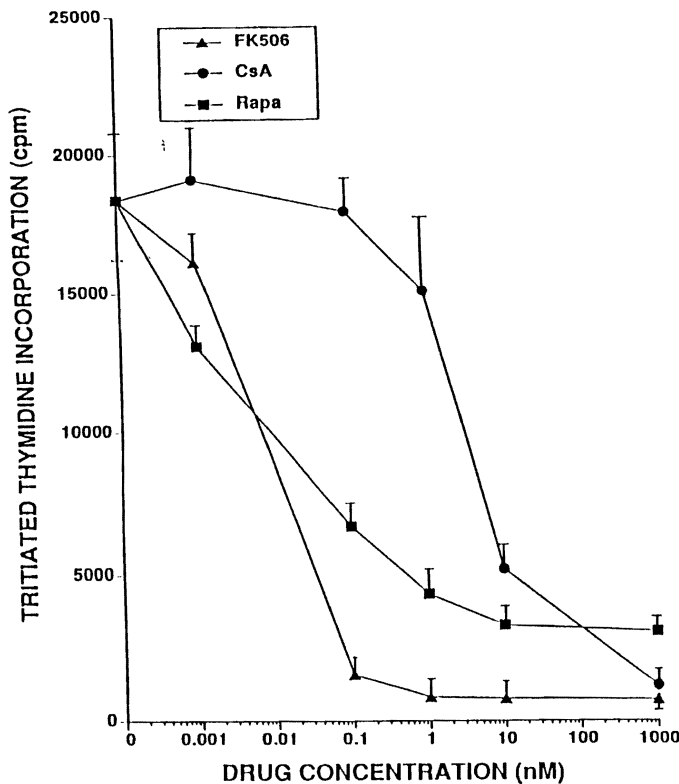


FIGURE 4. Rapamycin, FK506, and CsA inhibit the proliferation of Con A-stimulated splenocytes. Splenocytes (1×10^5 /6-mm well) were cultured for 3 days in the presence or absence of 1 μ g/ml Con A and increasing concentrations of CsA, FK506, or rapamycin. The cells were pulsed with 1 μ Ci/well [3 H]thymidine during the last 18 hr of the culture period. Data are the means \pm SD cpm incorporated by cells in 4 identical wells in an experiment representative of 3 similar experiments. Values obtained for cells treated with concentrations of: rapamycin $\geq 10^{-3}$ nM; FK506 ≥ 0.1 nM; and CsA ≥ 10 nM are statistically less than the values obtained for the untreated controls ($P < 0.005$).

to those that inhibited the blastogenic response of mouse splenocytes to Con A. Similarly, rapamycin blocked the proliferation of BMMP cultured in the presence of rhM-CSF, rmGM-CSF, and rmIL-3. In contrast, FK506 and CsA failed to inhibit the proliferation of BMMP at drug concentrations that inhibited Con A-induced blastogenesis of splenocytes. None of the 3 immunosuppressants tested prohibited the death of BMMP cultured in the absence of CSFs. This latter finding suggests that the inhibitory effect of rapamycin on the proliferation of BMMP was exerted specifically on the response of cells to CSFs.

These findings correlate with published reports demonstrating that different mechanisms underlie the effects of FK506 and CsA compared with rapamycin on the activation or proliferation of T lymphocytes (12, 13). FK506 and CsA inhibit the early, calcium-dependent events in T cell activation that occur subsequent to antigen-TCR interactions (30). These early events are distinguished by the accumulation of intracellular calcium leading to the elevated expression of early phase activation genes which encode growth-promoting cytokines such as IL-2 and IL-4 (15). While both FK506 and CsA inhibit the production of IL-2 and IL-4 by T lymphocytes,

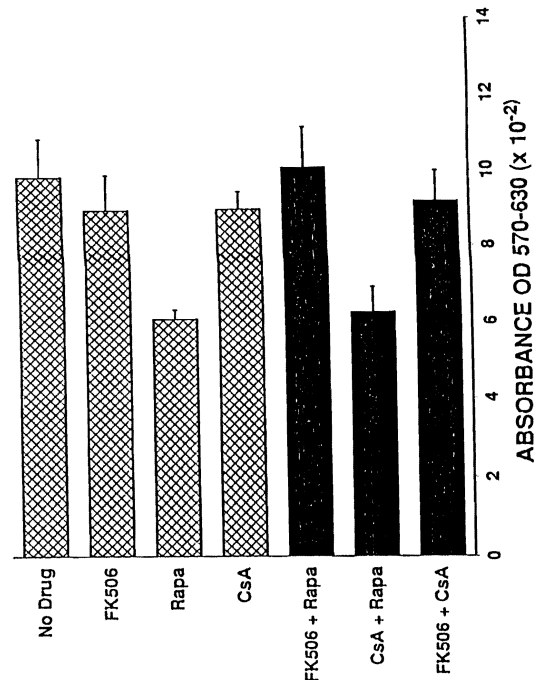


FIGURE 5. FK506 reverses the inhibitory effect of rapamycin on BMMP proliferation. BMMP (5×10^3 /well) were cultured in the presence of 10% LCM with or without CsA, FK506, or rapamycin (10 nM final concentrations) added singularly or in the combinations shown. Cell proliferation was assessed by MTT metabolism on the fifth day of culture. Data are the means \pm SD OD₅₇₀₋₆₃₀ obtained from quadruplicate wells in a single experiment. Comparable results were obtained in 2 similar experiments. The values obtained for cells incubated with rapamycin alone or in combination with CsA are significantly less than the values obtained for untreated control cells ($P < 0.001$).

neither impairs the calcium-independent proliferative response of cells to such cytokines (13). Rapamycin, on the other hand, inhibits the response of T cells to IL-2 and IL-4 without blocking cytokine production. Thus, it has been suggested that complexes formed between rapamycin and its cytosolic receptor preferentially block the signal transduction pathways used by growth factors (19).

FK506, CsA, and rapamycin exert effects on other immune cells that are similar to their effects on T lymphocytes. For example, FK506 and CsA inhibit the antigen receptor-mediated, calcium-dependent activation of B lymphocytes, but not the calcium-independent proliferative response of B lymphocytes to mitogens such as LPS or 8-mercaptopguanosine (31, 32). Similarly, FK506 and CsA inhibit the IgE receptor-mediated exocytosis of pharmacological mediators by human mast cells (33-35). Rapamycin, on the other hand, blocks the mitogenic response of B lymphocytes to LPS and 8-mercaptopguanosine, and has no effect on receptor-mediated exocytosis exhibited by mast cells.

The contrasting effects of FK506 and rapamycin on cell activation are somewhat surprising in view of their structural similarity and their apparent affinity for the same intracellular binding protein, FKBP (30). The affinity of FK506 and rapamycin for the same cytosolic receptor was first suggested by studies demonstrating their capacity to act as reciprocal antagonists during the activation of murine T lymphocytes.

phocytes (13). It was later found that rapamycin readily displaced FK506 from FKBP in a competitive binding assay (36). More recent studies suggested that FK506 and rapamycin also compete for a common intracellular receptor in B lymphocytes (31) and mast cells (34). Similarly, in the study reported here, FK506 reversed the inhibitory effect of rapamycin on the proliferative response of BMMP to CSFs, suggesting that FK506 and rapamycin may compete for a common intracellular receptor in BMMP. CsA did not prevent rapamycin-mediated suppression of BMMP proliferation. This is consistent with its interaction with an entirely different intracellular binding protein, cyclophilin (21, 22).

Further studies have helped to clarify the mechanisms that underlie the immunosuppressive effects of FK506, rapamycin, and CsA, and to resolve the different effects of FK506 and rapamycin on T cell activation. It is now believed that the biological activities of these immunosuppressants are mediated by complexes formed between the drugs and their intracellular binding proteins. For CsA and FK506, these complexes (CsA-cyclophilin and FK506-FKBP, respectively) bind and inactivate calcineurin, a protein phosphatase that plays a critical role in the early, calcium-dependent events that culminate in T cell activation (37, 38). In this regard, it is relevant to note that the mitogenic response of BMMP to the CSFs occurs in the apparent absence of calcium involvement (39). Thus, the failure of FK506 and CsA to suppress the proliferative response of BMMP to the CSFs implies that calcineurin is not a critical component of the activation pathway. While rapamycin binds to the same cytosolic receptor as FK506, available data suggest that the rapamycin-FKBP complex interacts with a molecule(s) other than calcineurin affecting the activation of T lymphocytes by calcium-independent mechanisms, e.g., those that mediate the response of cells to cytokines such as IL-2 and IL-4 (38). Our findings indicate that the proliferative response of BMMP to CSFs, similar to the response of T lymphocytes to IL-2 or IL-4, is mediated by a Ca^{++} /calcineurin-independent pathway.

The findings reported here have important implications in view of the potential use of rapamycin, FK506, and CsA in a general clinical setting. Based upon experimental data demonstrating the efficacy of FK506 in preventing organ allograft rejection (11), autoimmune diseases (8), and graft-versus-host disease after allogeneic BMT (40), clinical trials involving FK506 are proceeding in the United States and Europe. Clinical use of rapamycin is presently restricted to dose-response trials conducted only in certain centers. Since the proliferative response of BMMP to CSFs is an integral feature of hematopoiesis and host defenses (24), it is more likely that patients receiving rapamycin would be compromised in their abilities to generate a functional mononuclear phagocyte population and to respond to infection. On the other hand, the capacity of rapamycin to suppress BMMP proliferation could be used in the treatment of certain diseases, e.g., granulomatous diseases such as histiocytosis or leprosy, in which BMMP play an integral role in the pathogenesis. Thus, careful consideration given to the potent inhibitory effects of rapamycin on BMMP proliferation should allow rapamycin to be used in an efficacious manner.

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