Suppression of Allograft Rejection by Combined Treatment With CsA, FK506, and MPA

Until recently, formal pharmacological principles had rarely been applied to determine whether simultaneously administered immunosuppressants produce a net state of immunosuppression in graft recipients that is antagonistic, additive, or synergistic compared with immunosuppression caused by the individual administration of each agent. For many years, Berenbaum has decried the experimental design and data analysis from studies in transplantation, among other fields, that have led to incorrect conclusions concerning immunosuppressive drug-drug interactions.[101] Several years ago, when we converted the standard mouse ear-heart transplant technique to a quantal bioassay, we tried to redress the inadequacies of previous studies of combination immunosuppressive therapy.[17]

When we first began investigating the immunosuppressive activity of RPM in vivo, we assumed that the structural similarity between RPM and FK506 predicted that both drugs affected the immune system very similarly because at that time there was no in vivo or in vitro data to the contrary. This assumption, combined with our previous finding that treatment with FK506 does not antagonize CsA immunosuppression in vivo,[17] led us to treat rat heart allograft recipients with minimally effective doses of RPM plus CsA.[3] This study was not sufficiently rigorous to enable us to conclude from our data that these two drugs interact to produce immunosuppression that is synergistic. However, we were able to show that this combination is not antagonistic and that the immunosuppression caused by this combined therapy is at least additive. A recent and extensive study[72] involving sixteen separate treatment groups that expanded previous work[91] clearly showed, that combined treatment with RPM plus CsA produces synergistic suppression of rat heart allograft rejection. A smaller subset of this study showed that combined treatment with RPM and CsA is also beneficial for the prolongation of rat kidney allografts.

Using the mouse ear-heart bioassay, we showed that multiple combinations of RPM plus CsA or RPM plus FK506 cause prolongation of graft survival that is synergistic as defined by isobologram analysis[6] when the treatment doses of each drug are less than their ED_{30}s. In addition, probit analysis has been used to show that combined treatment of mouse skin allograft recipients with RPM plus CsA produces immunosuppression that is synergistic.[35] Furthermore, high doses of both RPM and FK506 used in combination did not indicate in any way that either drug antagonizes the immunosuppressive effects of the other. The data showing that treatment with RPM plus FK506 produces synergistic immunosuppression at many dose levels contradict studies in vitro that showed these two drugs antagonize each other's effects on immune cells (discussed in section headed Effects of RPM on Immune Cells in Vitro).

Lately, we have extended our interest in combination immunosuppressive drug therapy to the combined use of three drugs that have different mechanisms of immunosuppressive action (Fig 3) and nonoverlapping toxicity.[9] For example, low-dose CsA, RPM, or MPA (administered as its prodrug, RS-61443) monotherapy ineffectively prolongs rat heart allograft survival (Table 11). When these same doses are used, but all three drugs are administered together, suppression of graft rejection is not only more effective than when each drug is used separately, but it is also more effective than when RPM plus MPA or CsA plus MPA is used. Additional follow- up is required to determine whether triple drug therapy is superior to treatment with RPM plus CsA.

RPM monotherapy of cynomolgus monkey heart allograft recipients prolongs graft survival (discussed previously). However, unlike the use of RPM in rodent graft recipients, monkeys seem more resistant to the immunosuppressive effects of RPM and more sensitive to its toxicity. Because RPM and CsA produce immunosuppression in rodent graft recipients that is synergistic, and because it is possible that the toxic effects of each drug are different, we treated monkey heart allograft recipients (Morris RE, Wang J, Shorthouse R, et al: unpublished observation, 1991) with low doses of both drugs for the first 100 days posttransplant (Table 10). This treatment regimen suppressed rejection much more effectively than treatment with either CsA or RPM monotherapy. Only two of the five monkeys treated with combination therapy rejected their heart allografts during the treatment period; all animals remained clinically well. Pharmacokinetic analyses of CsA blood levels showed that the 2 mg/kg dose of CsA produces CsA levels that are subtherapeutic (all <60 ng/mL). Concomitant treatment with RPM and CsA does not elevate CsA blood levels compared with levels attained with CsA treatment alone. Thus, the im-

Table 11. Effect of Combination Therapy with RPM, CsA and Mycophenolic Acid (as its morpholoethylester, RS-61401) on the Survival of Brown-Norway Rat Heterotopic Abdominal Heart Grafts Transplanted into Lewis Recipients

Drug(s)	*Dose (mg/kg)*	*Route*	*Formulation*	*Schedule (days)*	*Median Graft (days)*
CsA	0.5	IP	Solution in Cremophor EL/ethanol	1 to 50	9
CsA	0.75	IP	Solution in Cremophor EL/ethanol	1 to 50	15
MPA	10	PO	Suspension in carboxymethyl cellulose	1 to 50	10
RPM	1.5	PO	Suspension in carboxymethyl cellulose	1 to 50	11
RPM	1.5	PO	Suspension in carboxymethyl cellulose	1 to 50	
+ MPA	10	PO	Suspension in carboxymethyl cellulose	1 to 50	28
CsA	0.75	IP	Solution in Cremophor EL/ethanol	1 to 50	
+ MPA	10	PO	Suspension in carboxymethyl cellulose	1 to 50	67
RPM	1.5	PO	Suspension in carboxymethyl cellulose	1 to 50	
+ CsA	0.5	IP	Solution in Cremophor EL/ethanol	1 to 50	109
CsA	0.5	IP	Solution in Cremophor EL/ethanol	1 to 50	
+ MPA	10	PO	Suspension in carboxymethyl cellulose	1 to 50	
+ RPM	1.5	PO	Suspension in carboxymethyl cellulose	1 to 50	170

proved immunosuppressive efficacy caused by combination therapy cannot be explained by high CsA blood levels (data not shown). The coadministration of CsA could elevate RPM levels, but without a blood level assay for RPM, this possibility cannot be examined. In the rat, coadministration of RPM and CsA does not elevate CsA levels.[61]

FK506 is known to suppress hepatic cytochrome P450 and the activities of ethylmorphine N-demethylase and cytochrome c reductase in rats,[102] and this may partly account for the increased half-life of CsA in patients treated with FK506.[67] Because we did not find that RPM increases the half-life of CsA in monkeys,[9] the effects of FK506 and RPM on the metabolism of CsA may differ. The interactions between nonimmunosuppressive macrolide antibiotics and CsA have been defined and may provide additional clues to the interaction between RPM and CsA.[103]

These initial studies of the lack of effect of treatment with RPM on CsA blood levels suggest that the combined use of RPM plus CsA may afford the benefits of increased immunosuppressive efficacy without the penalty of decreased safety. In view of the similar mechanisms of immunosuppressive action of CsA and FK506, the superior efficacy and potency of FK506, and the synergistic immunosuppression caused by the administration of RPM plus FK506 in mouse ear-heart recipients,[6] the combined use of RPM and FK506 may also be useful in monkey and human graft recipients. However, the increased nephrotoxicity and diabetogenic effect of combined high doses of RPM plus CsA in the rat[68] alerts us to the possible synergistic toxicity that can be caused by specific drug combinations.

Interactions Between RPM and Nonimmunosuppressive Drugs

RPM is likely to be used in patients receiving complicated treatment involving a wide variety of nonimmunosuppressive drugs. In addition to the interactions of RPM with other immmunosuppressants (previously discussed), coadministration of nonimmunosuppressive drugs may also substantially influence our goal of optimizing the dose, route, and schedule of administration of RPM. Because RPM shares some physicochemical characteristics with CsA, and is structurally similar to FK506, the extensive experience of CsA drug interactions[103] and the increasing understanding of the pharmacology of FK506[67,101] may provide lessons that will not have to be completely relearned with RPM. If, like CsA and FK506, RPM blood levels and its pharmacological effects vary widely among patients, and if the therapeutic index of RPM is low, the effects of simultaneously administered drugs will profoundly affect the clinical use of RPM.

As described in a recent review on CsA drug interactions,[103] this potentially complex problem can be simplified, at least conceptually, by analyzing how drug interactions affect the absorption, distribution, metabolism, and elimination (pharmacokinetics) of drugs and their the biolocial/toxicological effects

(pharmacodynamics). For example, coadministration of drugs that affect bile flow and gastrointestinal dysfunction should affect the absorption of orally administered RPM. The physicochemical similarities among RPM, CsA, and FK506 also suggest that the distribution of RPM in tissues and cells will be similar to CsA and FK506. Although the metabolic pathways for RPM are unknown, the structural similarity of RPM with FK506 would suggest that RPM is primarily metabolized in the liver. Once the metabolic pathways in the liver for RPM have been defined, we will be able to predict how other drugs might alter its metabolism. For example, there is extensive information on how inhibitors and inducers of hepatic enzymes associated with the cytochrome P450 system affect the metabolism of CsA.[103]

CsA and FK506 are nephrotoxic at immunosuppressive doses, and RPM is not completely without the potential to cause nephrotoxicity.[68] Therefore, drugs that are known to exacerbate CsA- and FK506-induced nephrotoxicity[103] should also be evaluated for their ability to unmask nephrotoxic effects of RPM.

Effects of RPM on Cells and Tissues of the Immune System in Vivo

For RPM to suppress allograft rejection or autoimmune diseases effectively, it must alter the normal functions of the immune system. Several studies using the most advanced techniques in cellular immunology and molecular biology have been intelligently exploited to try to understand how RPM affects immune cells under rigidly defined conditions in vitro. On one hand, these highly controlled experimental systems provide relatively clean answers to significant questions about the effects of a drug on very specific immune functions; on the other hand, important drug effects that fall outside the necessarily narrow focus of these investigations can be completely overlooked. Even when in vitro studies are focused appropriately, the answers that these experiments provide may not always be relevant to mechanisms of immunosuppressant drug action in vivo.[23] Changes in drug blood level, drug binding to plasma proteins, conversion of the parent drug into active and inactive metabolites, and the complex microenvironment of fluctuating cytokine levels that characterizes the response of the immune system to antigen in vivo cannot be duplicated in vitro. Therefore, before examining the effects of RPM on approximations of the immune system in vitro, we will review what little is known about the effects of RPM on components of the immune system in vivo.

Suppression of the Host-Versus-Graft and Graft-Versus-Host Responses

The host-versus-graft (HvG) popliteal lymph node (PLN) assay approximates the mixed lymphocyte reaction in vitro. We found that treatment with RPM, CsA, or FK506 suppresses the increase in PLN weight caused by the injection of irradiated BALB/c spleen cells into the hind feet of C3H mice.[6] Ongoing studies using flow cytometric and in situ hybridization analyses are designed to determine whether these drugs suppress the response of the PLN to alloantigen by inhibiting cytokine gene transcription, cytokine synthesis, cell proliferation, migration of cells into the node, or by promoting the exit of cells from the node (Morris RE, Shorthouse R, Zheng B, et al: unpublished observations, 1991). Despite the superior potency of RPM in the mouse ear-heart bioassay, RPM inhibits the HvG response less potently than FK506. This finding suggests that the exceptional efficacy of RPM for prolongation of murine heart graft survival may be more complex than can be accounted for by the PLN assay.

Recently, the in vitro function of cells in PLN draining the foot pads of mice that had been injected with allogeneic cells has been examined.[105] In contrast to mice treated with CsA or FK506, the PLN cells from mice treated with RPM incorporate less thymidine spontaneously or when stimulated with interleukin-2 (IL-2). Cells from RPM-treated mice also are less capable of generating cytotoxic T-cell activity or natural killer cell activity than cells from PLN from mice treated with either CsA or FK506.

The PLN assay can also be used to approximate the graft-versus-host response (GvH). We found that RPM is able to suppress this response in mice.[6] These results suggest that RPM may have the potential to control this disease in recipients of allogeneic bone marrow transplants. Further speculation led us to propose that RPM be used to facilitate engraftment of bone marrow derived from organ donors and for the creation of a chimeric state for induction of donor-specific unresponsiveness in human graft recipients.[6]

Effects on Numbers and Immune Function of Peripheral Blood T and B Cells

As part of a subchronic toxicity study of RPM in mice (unpublished), total WBC counts were monitored in mice that had been treated IP daily for 14 days with a

very high dose (24 mg/kg) of RPM in suspension. The WBC counts are normal on day 14, but during the recovery period it was found that this dose of RPM depresses the WBC counts. However, after 2 weeks of daily IP treatment of rat heart recipients with 6 mg/kg of RPM (a dose that produces > 200 day graft survival for all grafts), the WBC and the total lymphocyte counts are normal. Thus, indefinite prolongation of graft survival in rats occurs after brief RPM treatment without depletion of circulating lymphocytes. Although RPM can moderately suppress the WBC count in mice, this effect is by no means sufficient to explain how RPM induces indefinite prolongation of graft survival. The rejection of third-party grafts transplanted into recipients bearing viable primary grafts (discussed in section headed Effects of RPM on Graft and Tissue Rejection) not only indicates that there are sufficient numbers of circulating lymphocytes to mediate rejection, but also indicates that these cells are selectively immunocompetent.

Within 2 weeks of treating monkeys with RPM monotherapy or with combination therapy of RPM plus CsA, the absolute lymphocyte count is lowered (Morris RE, Wang J, Zheng B, et al: unpublished observations, 1991). Flow cytometric analysis of preparations of monkey whole blood showed (Morris RE, Wang J, Zheng B, et al: unpublished observations, 1991) that the total numbers of both T and B cells are lower than pretreatment values. In untreated monkeys, the ratio of the number of CD8+:CD4+ cells is greater than unity. RPM treatment causes this ratio to become inverted, because there is a disproportionate reduction in the number of CD8+ cells compared with CD4+ cells. It does not seem that the alterations in cell number alone can account for the suppression of graft rejection caused by RPM. More likely, RPM produces immunosuppression by functionally inactivating immune cells. For example, when we quantitated the response of peripheral blood mononuclear cells in monkeys treated with RPM plus CsA to different concentrations of concanavalen A (ConA) in vitro, we found that mitogenic responses are suppressed at low concentrations of ConA but return to levels similar to pretreatment values as the concentration of ConA in culture is increased (Morris RE, Wang J, Zheng B, et al: unpublished observations, 1991). Changes in the numbers and function of circulating peripheral blood B cells, T cells, and T-cell subsets caused by RPM in the monkey may also occur in humans treated with RPM. If so, these parameters may provide a more sensitive index of the effects of RPM on the immune system than assessment of graft rejection.

Effects on the Morphology and Function of Central Lymphoid Tissue

The only hint of the rationale for the first investigation of the immunosuppressive effects of RPM by Martel et al[19] was a brief sentence in the Discussion section of their article which read, "...long-term toxicity studies in dogs (Hemm RD, Authier L: personal communication) have demonstrated that rapamycin caused hypoplasia of lymphatic tissues (lymph nodes, spleen, and thymus)." This effect of RPM has now been confirmed in other species. For example, as part of an initial subchronic toxicology study we treated mice IP daily with a dose of RPM (24 mg/kg) that far exceeds doses (6 mg/kg) required to prolong ear-heart grafts indefinitely. Necropsies (Morris RE: unpublished observations, 1989) of mice on day 14 showed the thymus to be dramatically involuted, but the lymph nodes and spleen seemed normal in size and weight. Microscopic analysis of the lymph nodes and spleen did not show any abnormalities, but the normal distinction of the thymic cortex from the medulla was often absent and thymic lymphoid depletion was profound. When other animals from the same treatment group were necropsied on day 25 (2 weeks after the last RPM dose), thymic involution persisted and lymphoid cells were decreased in the medullary cords of lymph nodes.

A more thorough study[20] was conducted in mice that were treated IP daily with 6 mg or .75 mg/kg of RPM for a maximum of 13 days. These mice and aged-matched control mice were necropsied on days 7, 14, 21, 42, and 102, and their thymus and spleen weights recorded. Tissues from the thymus and spleen were stained with monoclonal antibodies and analyzed by immunohistochemistry and flow cytometry. Finally, spleen cells were cultured and stimulated with increasing concentrations of either the T-cell mitogen ConA, or the B-cell mitogen *Salmonella typhimurium* (STM).

We found that RPM treatment does not decrease the weight of the spleen. In contrast, RPM treatment has complex effects on the weight of the thymus.[20] The 6 mg/kg dose of RPM causes the thmus weight to be reduced by 80% after 1 week of treatment. The thymus weight increases, but is still abnormally low by day 42; by day 102, the weight rebounds to normal. The lower dose of RPM prolongs survival graft less effectively than does the high dose, but reduces

thymus weight by 57%. In these animals, thymus weight recovers more rapidly than in mice treated with the higher RPM dose. The positive correlations between RPM dose and prolongation of heart graft survival and between dose and the duration of thymus weight loss suggests that the effects of RPM on the thymus contribute to its immunosuppressive efficacy in the mouse.

In contrast to the effects of treatment with high- or low-dose RPM, treatment of mice with 6 mg/kg FK506 produces thymus weight loss of only 20%. This dose of FK506 prolongs the survival of ear-heart grafts longer than low-dose RPM, suggesting that involution of the thymus may be more critical to the immunosuppressive efficacy of RPM than for the efficacy of FK506.

Microscopic analysis of thymic tissue from RPM-treated mice is not complete.[88] Preliminary results from hematoxylin and eosin staining show that RPM treatment disrupts the normal thymic architecture. The changes in the cortex and medulla are variable, but suggest that thymocytes from both areas are depleted. Immunohistochemical staining with monoclonal antibodies directed to the pan T cell and helper and suppressor/cytotoxic phenotypes has shown that RPM causes T-cell depletion; $CD4^+$ and $CD8^+$ thymocytes stain especially weakly in the deep cortex. These studies have been performed in collaboration with investigators at Stanford University, Stanford, CA and with W.E. Beschorner at Johns Hopkins University, Baltimore, MD. Recently, we have found that the cortex is depleted in rats treated IV with high doses of RPM. For reasons that are unclear, this effect differs from the thymic medullary atrophy caused by treatment with lower doses of RPM.[68]

The high frequency of $CD4^+/CD8^+$ double-positive thymocytes in the normal thymus makes interpretation of the immunohistochemical staining with single antibodies difficult. Therefore, to learn more about the effects of RPM on thymocytes we have begun to use two-color flow cytometry. The results of these studies are complex and cannot yet be discussed in detail.[88] In brief, the percentage of $CD4^+/CD8^+$ cells is decreased by RPM treatment compared with age-matched controls and reaches a nadir on day 14. Although the percentage of this thymocyte subset recovers after cessation of RPM treatment, it is still statistically less than normal on day 42. RPM also causes a coincident increase in the percent of double- and single-negative ($CD4^-/CD8^-$ and $CD4^+/CD8^-$, $CD4^-/CD^+$) thymocytes; these increases probably represent a relative, rather than an absolute, increase caused by the primary effect of double-positive thymocyte depletion. FK506 treatment causes an effect that is the opposite of RPM: the percentage of double-positive cells increases and the percentage of single-positive thymocytes decreases.

Treatment of mice with RPM also produces complex changes in the response of spleen cells to T- and B-cell mitogens in culture[88] (Morris RE, Shorthouse R: unpublished observations, 1990). Even after cell washing in preparation for culture, spleen cells are hyporesponsive to stimulation with STM during and after treatment. By day 42, this response returns to normal. Thus, the antiproliferative effects of RPM are not solely restricted to T cells. In contrast to the effects of RPM treatment on the response of spleen cells to STM, the response to ConA is not suppressed. In fact, spleen cells from RPM-treated mice seem to be more responsive to ConA than spleen cells from age-matched control mice. Furthermore, this hyperresponsiveness increases with time after the cessation of RPM treatment.

Recently, we have found that RPM treatment also suppresses the increase in PLN weight caused by the injection of STM into the hind foot pads of mice (Morris RE, Shorthouse R: unpublished observation, 1991). These results confirm the finding that RPM treatment of mice suppresses the response of spleen cells in vitro to stimulation by STM. When succinylated ConA is injected, RPM treatment suppresses the PLN response (Morris RE, Shorthouse R: unpublished observations, 1991). Taken together, the data from all PLN assays (HvG, GvH and stimulation by T- or B-cell mitogens) show that RPM suppresses activation of immune cells stimulated by a variety of activation signals in vivo. Thus, RPM seems to cause at least two major effects that contribute to its immunosuppressive efficacy: (1) reversible depletion of immature thymocytes; and (2) functional inactivation, but not substantial depletion, of lymph node and spleen cells. The hyperresponsiveness of spleen cells to stimulation by ConA in vitro long after RPM treatment has ceased may be caused by immature T cells emigrating from the regenerating thymus to the spleen.

Since the original comment by Martel et al,[49] the effects of RPM treatment on central lymphoid tissue in large animals have been described by other investigators.[69,72] In our study of monkeys treated for long periods with immunosuppressive doses of RPM (discussed in section headed Effects of RPMs on Graft

and Tissue Rejection), there was some depletion of cells in lymph nodes, but the histology of the spleen was essentially normal. Because these monkeys were juveniles when their RPM treatment began, it was difficult to ascribe the lack of thymus tissue at necropsy entirely to the effects of RPM.

The investigations of the effects of RPM on the composition of murine thymus and spleen and the immune function of cells from the spleen and lymph node have just barely begun to scratch the surface of the complex effects of RPM on tissues of the immune system. Despite our present naivete, available data show that RPM affects central lymphoid tissues differently than CsA and FK506. Both CsA[106-109] and FK506[110-113] cause less thymic involution and affect the thymus more selectively than RPM because their effects are restricted to depletion of thymocytes in the medulla and not the cortex. Brief treatment with either CsA or FK506 at doses that exceed those needed for immunosuppression causes reversible thymus weight loss. Prolonged treatment with CsA renders the thymus incapable of recovery. Both CsA and FK506 seem to mediate their effects directly or indirectly by damaging medullary epithelium. This effect, perhaps in addition to others, may interrupt the maturation of single-positive $CD4^+/CD8^-$, $CD4^-/CD8^+$ thymocyte subsets from their double-positive precursors, cause an increase in the proportion of double-positive cells, and reduce the migration of cells from the cortex to the medulla.

Although the unusual effects of RPM on the morphology and thymocyte subset composition cannot be fully explained until more is known about its actions, we can speculate on mechanisms that might be responsible for the effects of RPM (Fig 8). If RPM treatment does not interfere with clonal deletion of double-positive cells by apoptosis but does block the rescue of double-positive thymocytes from cell death (positive selection), a net loss of double-positive thymocytes cells will occur. Treatment with FK506 (or CsA) will have the opposite effect because FK506 (or CsA) will prevent negative selection of potentially autoreactive double-positive cells by programmed cell death. This tentative hypothesis may explain why animals briefly treated with RPM do not develop the syndrome of syngeneic GvH (Zheng B, Morris RE: unpublished observation, 1991) that has been described in animals treated with CsA.[108,111] Three lines of evidence[115-117] support this hypothesis: (1) RPM does not inhibit activation-induced hybridoma apoptosis and cell death in vitro; (2) CsA inhibits DNA fragmentation in immature thymocytes, and FK506 inhibits activation-induced apoptosis of hybridoma cells; and (3) recently it has been suggested that the

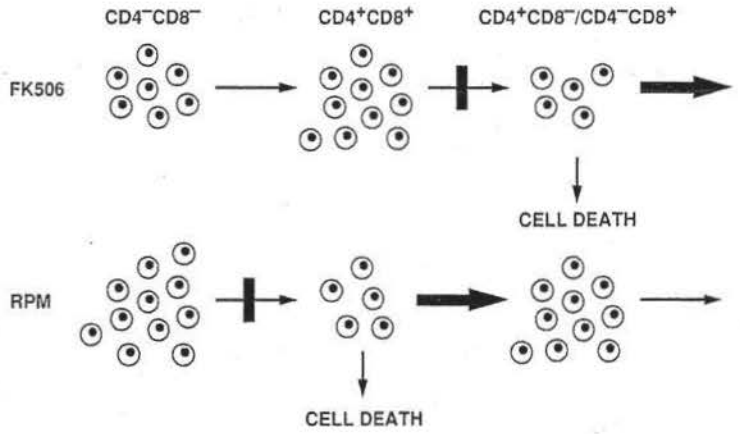

Figure 8. Possible effects of treatment with FK506 or RPM on the intrathymic differentiation of thymocytes. Flow cytometric analyses of thymocytes from FK506-treated mice and RPM-treated mice show decreased proportions of single-positive (CD4+CD8−/CD4−CD8+) and double-positive cells (CD4+CD8+), respectively. Although other explanations are possible (increased cell death of single-positive cells or their accelerated migration [*bold arrow*] into the periphery), the effect of FK506 treatment is most likely caused by an interruption in the maturation of double-positive thymocytes. Failure to be rescued from positive selection resulting in increased cell death is the most likely explanation for the decrease in double-positive thymocytes in mice treated with RPM; an interruption of maturation from double-negative cells or an accelerated differentiation from double-positive cells into single-positive thymocytes could also explain the effects of RPM treatment.

CsA/FK506-resistant CD28 signal transduction pathway in thymocytes is necessary for positive selection in the thymus[118] and RPM inhibits lymphocyte activation in vitro via this pathway.[25,30,32,117,119]

Other phenomena might explain the effects of RPM treatment on thymocyte subpopulations. For example, RPM could be directly toxic to double-positive cells and cause increased cell death. A less likely explanation for the low percentage of double-positive cells is that RPM accelerates the differentiation of this immature population to cells bearing the more mature single-positive phenotype. Although the percentage of single-positive cells increases during RPM treatment, the substantial loss of cells in the thymus caused by RPM favors a net cell loss of thymocytes rather than their redistribution by accelerated maturation from the cortex to the medulla. Because the increase in the percent of double-negative thymocytes in RPM-treated mice is not quantitatively inversely proportional to the decrease in double-positive cells, it is unlikely that RPM causes a decrease in the percentage of the double-positive population solely by arresting the maturation of double-negative thymocytes to the double-positive phenotype.

Our preliminary findings on the distinct effects of RPM on murine thymocyte populations combined with our studies in vivo on the acquisition of specific unresponsiveness to ear-heart grafts suggests that RPM treatment may provide the appropriate environment for the induction of tolerance. RPM, acting on mature circulating T cells, could prevent immediate graft rejection. RPM, acting on thymocytes, could enable maturing and potentially alloreactive thymocytes in the recovering thymus to be negatively selected when donor major histocompatibility complex (MHC) peptides are presented by thymic dendritic cells in the context of self-MHC. However, we have recently found that RPM treatment of adult thymectomized recipients of ear-heart allografts also causes prolongation of graft survival (Morris RE, Shorthouse R: unpublished observations, 1991). Although prolongation of graft survival in these mice is not as great as in RPM-treated euthymic mice, any hypothesis of the mechanism of immunosuppression of RPM may have to be expanded to include clonal anergy and active suppression.

In the future, the pharmacological effects of RPM on the thymus and other primary lymphoid tissues may provide valuable clues to define the events leading to self and non-self discrimination. Perhaps a method will be found to use RPM to induce specific unresponsiveness in human graft recipients and in patients with autoimmune diseases.

Effects of RPM on Immune Cells In Vitro

The structural similarity between FK506 and RPM and the previously described immunosuppressive effects of RPM in vivo prompted the initiation of studies of the suppression of graft rejection of RPM in vivo and its effects on immune cells in vitro. The specific motives of the investigators evaluating RPM in vivo differed from those who included RPM in their in vitro experiments. RPM was the primary focus of the in vivo studies designed by investigators at the Laboratory of Transplantation Immunology of Stanford University to define its immunosuppressive efficacy and mechanisms of action; FK506 was included for comparison. FK506 was the primary focus of in vitro studies designed to define its mechanisms of immunosuppressive action; RPM was included for comparison. Regardless of the difference in motives for studying the effects of RPM on the immune system in vivo and in vitro, both approaches simultaneously showed that RPM and FK506 affect the immune system quite differently.

Despite the role of RPM as a supporting actor in most in vitro studies, its effects on immune cells would be far less clear had it been given no role at all. Other immunosuppressive drugs such as CsA have been used as tools to pry apart the biochemical components of T-cell activation in vitro and have initiated a self-perpetuating process of immunosuppressive drug discovery and development. For example, as the understanding of immune cell activation increases, it becomes easier to distinguish among differences in the mechanisms of action of new drugs like FK506 and RPM, and new strategies for immunosuppression emerge. It is difficult to pinpoint the precise mechanisms of immunosuppressive action of RPM from in vivo experiments because the drug effects of RPM in vivo are the net result of many unknown interactions among RPM, the immnune system, and other biological systems. In vitro experiments offer the opportunity to observe the effects of unmetabolized RPM on defined biochemical events during the controlled activation of well-characterized immune cells.

Despite these advantages of working in vitro, it can be treacherous to assume that the mechanisms of action of an immunosuppressant defined in vitro apply equally to its actions in vivo.[21] For example,

Table 12. Effects of Treatment with FK506 or RPM on T Cells In Vitro

In Vitro Activity Studied	*Cell Type*	*Activation Stimuli*	*Drug Effects* FK506	RPM
ACTIVATION				
Ca^{2+}-DEPENDENT				
Gene transcription				
NF-AT activity	Jurkat	None	⇊	ø
NF-AT-DNA binding	Jurkat	Ionomycin + PE	↓	ø
c-fos	Human PBL	ConA + PE	ø	ø
IL-2	Mouse spleen, human PBL, Jurkat	ConA + PE, Ionomycin + PE, anti-CD3 ± PE	⇊, ø	ø
IL-3, -4, GM-CSF, *c-myc*, TNF-α, IFN-γ	Human PBL	ConA + PE	⇊	ø
IL-2Rα, Tf-R, TNF-β	Human PBL	ConA + PE	ø	ø
Cytokine production				
IL-2	Mouse spleen, human PBL, mouse IL-2–producing hybridoma, Jurkat	ConA ± PE, Ionomycin + PE, anti-CD3 or anti-CD2 + PE	⇊	ø, ↓
IL-1	Human PBL	ConA	⇊	↓
IL-6	Human PBL	ConA	ø	↓
TGF-β	Human PBL	ConA	ø	ø
IFN-γ	Human PBL	ConA	⇊	⇊
IL-2R cell surface expression	Mouse spleen, human PBL	PE + Ionomycin, anti-CD3 or anti-CD2 + PE	ø	↓
DNA synthesis	Mouse spleen, human or porcine PBL, mouse Th2 clone	ConA or PHA ± PE, Ionomycin + PE, anti-CD3 or anti-CD2 + PE, anti-CD2 (T11.2) + anti-CD2 (T11.3), MLR	⇊	⇊
	Human primed lymphocytes from transplant biopsies	Allogeneic human PBL	⇊	↓
Protein synthesis	Human or porcine PBL	ConA, PHA, anti-CD3	⇊	⇊
Reversal of drug effect after drug wash out? Protein, DNA synthesis	Porcine PBL, mouse spleen	ConA ± PE	No, Yes	No, Yes
Block in cell cycle progression				
$G_o \rightarrow G_1$	Mouse spleen, human or porcine PBL	ConA, Ionomycin + PE, PHA, ConA	↓	ø
$G_1 \rightarrow S$	Human or porcine PBL		ø	↓
Inhibition of DNA synthesis in okadaic acid–treated cells during G_1	Mouse spleen		ø	↓
Apoptosis	Mouse hybridoma	Ionomycin + PE, anti-CD3	⇊	ø
Ca^{2+}-INDEPENDENT				
Cytokine production				
IFN-γ	YAC-1 lymphoma	IL-1 + PE	—	↓
Noncytokine-induced protein or DNA synthesis	Human or porcine PBL, alloactivated human PBL	Anti-CD28 + PE	ø	⇊
		PE	ø	⇊
Cytokine-induced protein or DNA synthesis	Human PBL, mouse spleen cells	IL-2 + PE, IL-2,	ø	⇊
	Activated human PBL			
	CTLL, porcine IL-2–dependent line	IL-2	ø	⇊
	Mouse Th2 clone	IL-1 + PE, IL-1 + Ionomycin	ø	⇊
	Mouse spleen cells, CTLL	IL-4 ± PE	ø	⇊
	IL-6–dependent line	IL-6	—	⇊
Cytokine-induced Ly-6E cell surface antigen induction	YAC-1 lymphoma	IFN-γ	ø	⇊
Cell mediated toxicity				
Generation of CTL	Human PBL	MLR	—	↓
pCTL frequency	Human PBL	MLR + IL-2	—	⇊
Constitutive DNA synthesis	Mouse hybridoma, Jurkat	None	ø	⇊
Constitutive protein synthesis	Porcine PBL	None	ø	↓
Cell viability	Mouse hybridoma, human PBL	None	ø	ø

Abbreviations: PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; PE: phorbal ester.
Symbols: —, not tested; ø, no effect; ↓, activity inhibited.

Table 13. Effects of Treatment with FK506 or RPM on B Cells and Basophils In Vitro

In Vitro Activity Studied	*Cell Type*	*Activation Stimuli*	*Drug Effects*	
			FK506	*RPM*
B CELLS				
ACTIVATION				
Ca^{2+}-DEPENDENT				
Protein synthesis	Mouse spleen	Anti-IgM	⇊	⇊
DNA synthesis	Mouse spleen	Anti-IgM, anti-IgM + IL-4	⇊	⇊
	Human PBL	PWM	—	⇊
Ia cell surface Ag expression	Mouse spleen	Anti-IgM	ø	ø
Block in cell cycle progression				
$G_0 \rightarrow G_1$	Mouse spleen	Anti-IgM	ø	ø
	Human PBL	PWM	—	↓
$G_1 \rightarrow S$	Mouse spleen	Anti-IgM	↓	↓
S	Mouse spleen	Anti-IgM	↓	ø
	Human PBL	PWM	—	ø
T cell-dependent Ig production	Human PBL	PWM	↓	↓
Ca^{2+}-INDEPENDENT				
Protein synthesis	Mouse spleen	LPS	ø	⇊
DNA synthesis	Mouse spleen	LPS, 8-mercapto-guanosine	ø	⇊
Viability	Mouse spleen	Anti-IgM	⇊	↓
Constitutive DNA synthesis	Daudi, human EB-transformed line	None	ø	⇊
BASOPHILS				
ACTIVATION				
Histamine release	Human PBL	Anti-IgE	⇊	↓
		A23187	⇊	ø

Abbreviation: LPS, lipopolysaccharide; PWM, pokeweed mitogen.
Symbols: —, not tested; ø, no effect; ↓, activity inhibited.

concentrations of RPM that are required to cause specific effects in vitro may be well in excess of the maximum tolerated plasma levels of RPM. The chemical instability of RPM in vitro (previously discussed) makes it impossible to know whether the parent or a degradation product is responsible for the effects observed. Complicating these problems is the lack of complete dose-response studies in some experiments. Furthermore, the contents of the culture medium (serum, cofactors, growth factors) and cell density can affect the observed effects of immunosuppressants in vitro. Finally, the activation signals used to stimulate immune cells in vitro may cause changes in second messengers along the signal transduction pathway that are quantitatively or temporally different from in vivo stimuli. Similar concerns apply to the in vivo relevance of data derived from the exposure of RPM to transformed cell lines.

Nevertheless, carefully selected information from the study of the effects of RPM on immune cells in vitro enables us to gain a deep understanding of the effects of RPM in vivo. In the final analysis, the sum of the knowledge of the in vivo and in vitro actions of RPM is far greater than conclusions derived from analyzing in vivo or in vitro data separately.

The subsequent section will review the results of published in vitro studies in which RPM was evaluated, and only the studies in which both FK506 and RPM were evaluated simultaneously in the same in vitro experimental systems. To date, the in vitro effects of RPM have been tested on cells from mice, pigs, and humans. These cells have included normal lymphocytes, hybridoma cell lines, transformed T and B cells, and normal basophils (Tables 12 and 13).

Effects of RPM on T Cells

Effects on T-cell activation. The effects of RPM on the activation of T cells, constitutive protein and DNA synthesis, cell-mediated toxicity, and cell viability have been reported (Table 12). The majority of these studies have concentrated on defining the effects of RPM on T-cell activation for several reasons: (1) the molecular events of this process are becoming increasingly clear; (2) FK506, the structural homologue of RPM, is known to inhibit activation; and (3) T-cell activation is an important compo-

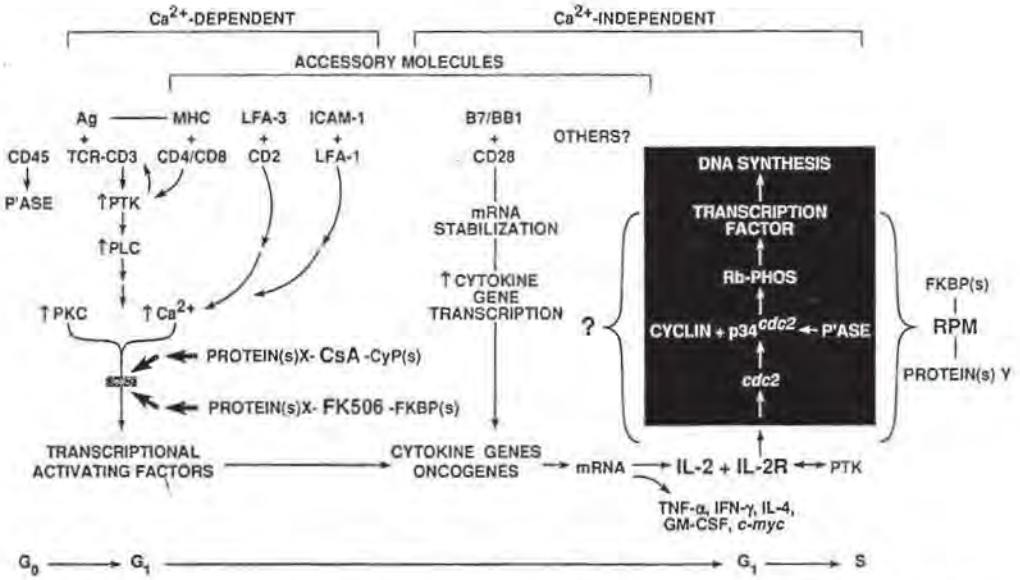

Figure 9. Biochemical pathways leading to T-cell proliferation after engagement of the TCR/CD3 complex with antigen and the interaction of accessory T-cell molecules with their ligands and IL-2 with IL-2R. The possible sites of action of the immunosuppressants complexed to cytoplasmic binding and effector proteins are shown: CsA bound to cyclophilins, FK506 and RPM bound to FKBP. Although not proven, it has been suggested that the effector proteins (proteins X) for CsA and FK506 are calcineurin/calmodulin. The effector proteins for RPM (proteins Y) have been neither identified nor shown to be required for the actions of RPM. Briefly, it is now believed that T cells require a minimum of two classes of signals before these cells are able to commit to proliferation after binding of IL-2 to IL-2R. In general, these signals are triggered by the interaction of an array of T-cell surface receptors with a variety of ligands and cytokines supplied by antigen-presenting cells. The first signal transduction pathway (signal 1) involves the binding of peptide antigen (complexed to MHC class I or II molecules) to the TCR/CD3 complex associated with CD4 or CD8 accessory molecules on T cells. The TCR on most mature T cells is comprised of the heterodimeric clonotypic α- and β-chains that are noncovalently associated with other transmembrane proteins of the CD3 complex (γ-, δ-, and ε-polypeptide chains) and the ζ- and η-chains. Ligation of the αβ-CD3-ζη complex causes ζ-chain phosphorylation by CD4- or CD8- and αβ-associated protein tyrosine kinases (PTK). Phosphatase activity, perhaps mediated by the CD45 transmembrane protein and other phosphotyrosine phosphatases, may also play roles in early and late activation events. PTK-dependent activation of phospholipase C causes the hydrolysis of polyphosphoinositides producing diacylglycerol and inositol 1, 4, 5-triphosphate. These events lead to the mobilization of intracellular and extracellular Ca^{2+} and protein kinase C and the change from resting T cells in G_0 to activated T cells in the G_1 phase of the cell cycle. As an indirect and incompletely understood consequence of these biochemical changes, transcriptional activating factors are translocated into the nucleus where they bind to enhancer regions of genes that code for cytokines (TNF, IFN, and GM-CSF) and oncogenes. Less well understood is the equally important role of costimulatory or second signals for T-cell activation. Acting in association with, and in some cases independent of, the transduction of signal 1 through the αβ-CD3-ζη complex, CD2 and LFA-1 cell surface accessory molecules bound to their respective ligands on antigen-presenting cells transduce second signals (signal 2) mediated by many of the same second messengers just discussed. Recently, the CD28 cell surface protein has been shown to interact with the B7/BB1 molecule on antigen-presenting cells to provide a separate and distinct avenue for costimulation. Other studies have shown that ligation of the CD28 receptor with anti-CD28 monoclonal antibodies combined with phorbol esters (direct protein kinase C inducers) causes production of IL-2 and cell proliferation that is resistant to inhibition by CsA and FK506. Unlike other signalling pathways, elevation of the intracellular Ca^{2+} concentration is not required for CD28 receptor-mediated signal transduction. Activation through the CD28 receptor causes enhanced transcription of cytokine genes and stabilization of cytokine mRNA. Thus, the second signal pathway provides for cytokine production in excess of that needed for autocrine stimulation and enables the immune response to be amplified by paracrine effects. Transcription of the IL-2 gene, translation of its mRNA into IL-2, and the binding of IL-2 to IL-2R are critical to the progression of T cells from G_1 to the S phase of the cell cycle. The precise events that occur after IL-2 binds to its receptor that lead to DNA synthesis and cell division are far from clear. Recently, a PTK has been described that associates within the β chain of IL-2R, thus enabling the IL-2R to initiate the signal transduction process. New information on the regulation of the mammalian cell cycle provides clues to the events necessary for cell cycle progression form G_1 to S. For example, stimulation of T cells induces thetranscription of the *cdc2* gene late in G_1. This gene codes for a serine-threonine protein kinase ($p34^{cdc2}$) that becomes

nent in the pathogenesis of graft rejection and autoimmune diseases.

The knowledge of the biochemical pathways that ultimately lead to T-cell proliferation[120-130] (Fig 9) has been exploited to begin to define where RPM acts. For example, T cells can be activated by a variety of defined stimuli (Table 12) that cause either Ca^{2+}-dependent or -independent activation. After stimulating T cells in these two different ways, it is then possible to study the effects of RPM on early events such as gene transcription and cytokine production, and later events such as IL-2–receptor expression and transcription of late phase T-activation genes, and DNA and protein synthesis. This information can be used to localize the inhibitory effects of RPM to particular stages of the cell cycle.

There is a clear difference between the effects of RPM and FK506 on early events after Ca^{2+}-dependent T-cell stimulation.[7,31,41] RPM does not inhibit early events (the transcriptional activity and DNA-binding of the transcriptional activator NF-AT, transcription of immediate early [*c-fos*] and most early phase T-cell activation genes (IL-2, -3, and -4, granulocyte macrophage-colony stimulating factor [GM-CSF], *c-myc*, tumor necrosis factor-α [TNF-α], and interferon-γ [IFN-γ]). In contrast, FK506 inhibits all these activities except for *c-fos* transcription[26-29,31,116,131]; one study failed to show that FK506 inhibits IL-2 gene transcription.[28] Neither FK506 nor RPM inhibits the transcription of late phase genes (IL-2Rα, transferrin-receptor [Tf-R], and TNF-β).[27] As expected from the different effects each drug has on transcription, most studies have shown that FK506 does and RPM does not inhibit the production of IL-2.[27,29,31,116,119,132] Investigators at the Laboratory for Transplantation Immunology at Stanford University and other institutions have found that RPM can inhibit IL-2 production,[26,79] and this may be related to differences among these studies concerning activation stimuli, assay times, and amount of IL-2 produced, or it may be an indirect result of the inhibitory effect of RPM on cell proliferation. We have studied the effects of FK506 and RPM on the synthesis of other cytokines secreted from human peripheral blood lymphocytes stimulated with ConA (Table 12).[79] As expected, FK506 inhibits IFN-γ production. RPM also inhibits the production of this cytokine. Therefore, the effects of RPM on early gene transcription may not be entirely predictive of the effects of RPM on cytokine secretion throughout the culture period. Neither RPM nor FK506 has stimulatory or inhibitory effects on tumor growth factor-β (TGF-β) production.

Because FK506 does not inhibit the transcription of the gene for IL-2R, it is not surprising that this cell surface receptor is expressed normally on cells treated with FK506. The lack of inhibition of RPM on IL-2R gene transcription and its clear inhibition of the expression of IL-2R on the cell surface reinforces the need for caution when extrapolating from the effects of RPM on transcription to its effects on translated protein.[26,119,131]

Regardless of the stimulus, both RPM and FK506 inhibit DNA and protein synthesis in activated T cells. Studies of the reversibility of inhibition of protein and DNA synthesis by RPM and FK506 produced mixed results. One study showed that washing cells after drug treatment failed to reverse the drug effect and another study indicated that removal of the drugs restored cells to normal.[25,26,28-30,79,119,131,133-136]

The effect of RPM or FK506 treatment on cell cycle progression was analyzed by adding these drugs to T cells at different times after initiation of activation and measuring protein or DNA synthesis.[26,28,30,136] These experiments showed that RPM allows the cells to proceed through G_1 but blocks progression to the S phase. In contrast, FK506 blocks only the transition from G_0 to G_1. Additional experiments have reinforced the belief that RPM and FK506 inhibit different stages of the cell cycle.[137,138] Okadaic acid is known to inhibit two serine/threonine phosphatases (PP1 and PP2A). Inhibition of the activity of these two phosphatases by okadaic acid does not inhibit early signal transduction in T cells and their transition from G_0 to G_1. Thus, okadaic acid and FK506 inhibit different stages of the cell cycle. The activity of these phosphatases must be crucial for cells to proceed to the S phase because okadaic acid prevents T cells from progressing from G_1 to S. When stimu-

←

activated when complexed with cyclin and when residues in its adenosine triphosphate binding site are dephosphorylated. The active $p34^{cdc2}$-cyclin heterodimeric complex forms the maturational promoting factor that seems to be involved in the progression of T cells from G_1 to S. The products of the *c-myc* and *c-myb* genes also seem to play important roles in the initiation of DNA synthesis. It has been proposed that the serine-threonine phosphorylation by $p34^{cdc2}$ kinase of pRb, the product of the retinoblastoma gene, is necessary to release a transcription factor from unphosphorylated pRb so this factor can promote the transition from G_1 to S. (Specific references cited in text.)

lated cells are treated with okadaic acid and allowed to enter G_1, treatment with FK506 does not inhibit DNA synthesis, but RPM does. These data suggest that RPM and okadaic acid inhibit events that occur at similar times in late G_1.

Through mechanisms not well understood, when mouse hybridoma cells are treated with stimuli that raise levels of intracellular Ca^{2+}, apoptosis occurs. FK506 inhibits this form of apoptosis, but RPM does not.[115-117]

Certain types of stimuli can produce T-cell activation measured by increases in protein or DNA synthesis that are independent of the need for increases in intracellular Ca^{2+}. RPM, unlike FK506, inhibits all types of Ca^{2+}-independent T cell activation. For example, the following activities are inhibited by RPM but not by FK506: (1) T-cell protein and DNA synthesis stimulated by antibody to the CD28 receptor or by phorbol esters; and (2) protein and DNA synthesis in T cells or IL-dependent cell lines stimulated by IL-2, -4, or -6, and IFN-γ–induced expression of the Ly-6E cell surface antigen.[25,26,29-31,117,119,131,133,141]

Other effects on T cells. RPM inhibits the frequency of precursor cytotoxic T cells more effectively than the generation of these cytotoxic cells[133] (Table 12). Unlike FK506, RPM inhibits constitutive DNA synthesis in mouse and human cell lines.[79,131] RPM, but not FK506, inhibits protein synthesis in resting lymphocytes.[30] The different inhibitory effects of RPM and FK506 previously described cannot be ascribed to cytotoxic effects because neither compound decreases the viability of mouse or human T cells.[116,131]

Effects on B Cells

Although the events leading to activation and proliferation of B cells are less well understood than for T cells, recent information indicates that both cell types rely on similar biochemical processes to transduce signals from membrane receptors to cytoplasmic second messengers shared by both classes of these cells.[140-142] Mature B cells express membrane-bound immunoglobulin (mIgM or mIgD) that binds antigen and interacts with two noncovalently associated, disulfide-linked heterodimeric proteins that are required for signal transduction. Characterization of these proteins has shown that mIgM is complexed with IgM-α and Ig-β (or less frequently, Ig-γ), and IgD is complexed with IgD-α and Ig-β (or less frequently, Ig-γ). Amino acid sequence analysis of IgM-α and Ig-β showed that these molecules are members of the Ig supergene family. Furthermore, the cytoplasmic tails of these proteins contain a conserved amino acid sequence motif that is shared by the the cytoplasmic tails of the γ, δ, and ζ chains of the T-cell receptor/cluster of differentiation 3 (TCR/CD3) complex. The suggestion that T and B cells use similar pathways for signal transduction is strengthened by the finding that the phosphotyrosine phosphatase membrane protein CD45 plays a critical role during B-cell activation.

With this limited understanding, a preliminary outline of the molecular interactions that participate in B-cell activation and proliferation has been proposed. Binding of antigen to membrane Ig receptors produces receptor crosslinking that causes a conformational change that activates a protein tyrosine kinase. The cytoplasmic tails of the αβ or αγ accessory molecules act as substrates for tyrosine phosphorylation. Phorphorylation and CD45-mediated dephosphorylation of these and other cytoplasmic proteins may trigger second messengers that lead to activation and ultimately to proliferation. Two activation pathways have been identified: (1) one leads to G-protein–dependent phospholipase C activation, changes in Ca^{2+} concentrations, and activation of both protein kinase C and Ca^{2+}-dependent kinase; and (2) another involves tyrosine phosphorylation of other substrates. There is also indirect evidence that underphosphorylation of serine and threonine residues of retinoblastoma gene products (pRb) can prevent B cells from progressing from late G_1 to the S phase. In the transition of T cells from late G_1 to S, pRb has also been implicated.

Measurement of protein, DNA, and Ig synthesis have been used to evaluate the effects of RPM and FK506 treatment on the activation of B cells by stimuli that operate through Ca^{2+}-dependent processes[30,136,139,143] (Table 13). Neither drug inhibits the increase in cell surface Ia antigen expression that occurs soon after B cells leave the G_0 phase of the cell cycle.[143] However, analysis of the effect of delayed addition of RPM on DNA synthesis of pokeweed mitogen (PWM)-stimulated cells showed that RPM inhibits only early events in the G_0-G_1 phase of the B-cell cycle.[136] Production of immunoglobulin by cultures stimulated with PWM is also potently suppressed by RPM.[139] Other studies of DNA synthesis, cell volume and DNA content in B cells stimulated by antibody-to-surface IgM indicated that RPM and FK506 treatment do not inhibit early postreceptor events involving transition from G_0 to G_1.[141] However, these same studies showed that whereas both drugs slow the progression of activated B cells through G_1, only FK506 inhibits B-cell progression in early S

phase. Rather than inhibiting proliferation, RPM delays it by acting at an earlier time in G_1 than FK506. The high proportion of dead cells in cultures of stimulated cells containing FK506 may have confounded the interpretation of the effects of FK506.

The results of the effects of treatment with RPM or FK506 on stimulation of B cells by Ca^{2+}-independent signals show that only RPM inhibits B cells activated by this pathway[30,136,143] (Table 13). Investigators at the Laboratory of Transplantation Immunology at Stanford University and other institutions have also found that RPM, but not FK506, inhibits the proliferation of B cell lines.[79,134]

Effects of RPM on Other Cells

In addition to its effects on T and B cells in vitro, the effects of RPM treatment on the release of histamine from human basophils has been studied.[81,85] Although RPM inhibits histamine release from basophil receptors crosslinked with anti-IgE antibody, the inhibition is only partial. FK506 is a far more effective inhibitor of histamine release in this system than RPM, and FK506 also inhibits histamine release from basophils treated with the calcium ionophore A23187. Interestingly, the cytoplasmic tails of the γ and β chains of the rat mast-cell receptor have the same amino acid motif as IgM-α and Ig-β proteins on B-cell receptors and certain proteins of the TCR/CD3 receptor complex previously discussed. Because CsA and FK506 inhibit granule exocytosis from cytotoxic lymphocytes and neutrophils, FK506 may affect basophils by similar mechanisms. Clearly, despite its structural similarity, RPM does not participate in this process.

RPM-Immunosuppressive Drug Interactions

Many studies have evaluated the effects of combining RPM with either CsA or FK506 on T- and B-cell and basophil functions in vitro.[28,30,31,84,119,131,135,144] Combined treatment with RPM and CsA produces additive or synergistic inhibition of protein and DNA synthesis in T cells activated by either the Ca^{2+}-dependent or-independent signal transduction pathways. Initial investigations of the effect of the combined use of both RPM and FK506 to inhibit T-cell activation by ConA showed that this combination produces inhibition that is either additive or antagonistic depending on the relative concentrations of each drug.[28] RPM and CsA additively suppress the expression of IL-2 receptor expression on activated T cells. Later, this phenomenon was examined more closely and it was found that equimolar concentrations of RPM and FK506 cause additive inhibition of ConA-activated T-cell proliferation, whereas these drugs become reciprocally antagonistic when either compound is present in a concentration that is in great molar excess (100×) over the other. There is no reciprocal antagonism between CsA and RPM, but the reciprocal antagonism between RPM and FK506 has been noted in other systems when either compound is used at much higher concentrations than the other. For example, the combined use of excess RPM plus FK506 inhibits the ability of FK506 to suppress NF-AT transcriptional and DNA-binding activity, transcription of early phase T-cell activation genes, IL-2 secretion, T-cell apoptosis, and calcium ionophore-mediated histamine release from basophils. Similarly, an excess of FK506 plus RPM inhibits the ability of RPM to suppress Ca^{2+}-independent T-cell and B-cell proliferation and cytokine-induced T- cell proliferation.

The effects of RPM on certain functions of a limited variety of immune cells have been well defined. This is because of the large body of results from in vitro experiments that have been reported in the last 2 years, and also because many investigators have evaluated the same in vitro phenomena using cells from the same or different species. Despite the potential for artifact in these in vitro studies, the conclusions concerning the effects of RPM on immune cells have been remarkably consistent among experiments performed by different investigators.

For the most part, these in vitro results conform to the effects of RPM on the immune system in vivo. RPM does not seem to be profoundly cytotoxic to mature lymphoid cells in vitro or in vivo, but rather it suppresses T- and B-cell proliferation to alloantigens and to T- and B-cell mitogens in vivo and in vitro. Evidence that RPM is an especially effective inhibitor of T-cell activation by costimulatory molecules may be highly relevant to its ability to alter intrathymic T-cell differentiation and may confer a unique ability to induce peripheral anergy. These quantities might be among the reasons that RPM so effectively produces long-term antigen-specific unresponsiveness.

The counterpart to the weak in vivo antiinflammatory effects of RPM is its relative lack of efficacy for the inhibition of histamine release from basophils. Taken together, the results from these in vivo and in vitro experiments make it unlikely that the ability of RPM to halt and reverse ongoing rejection is because of its antiinflammatory effects. It would be more reasonable to ascribe the suppression of ongoing

rejection to the ability of RPM to inhibit T- and B-cell activation by both Ca^{2+}-dependent and -independent pathways. Furthermore, in addition to the inhibition of cytokine-induced proliferation by RPM, it also may be able to suppress other cytokine effects (upregulation of cell surface receptors, class II antigens, adhesion molecules, cytokine-induced cytotoxicity) that contribute to the rejection response.

Regardless of which drugs are used, it is unlikely that accurate predictions of how combinations of immunosuppressive drugs will interact in vivo can be deduced solely from in vitro assays of immunosuppressive drug-drug interaction. In addition to the more general limitations of in vitro assays previously discussed in this section, there are specific concerns about the interpretation of results from in vitro systems in which combinations of immunosuppressive drugs are used. For example, the relative concentrations of the drugs are likely to have a significant impact on the net immunosuppressive effect of combination therapy. The blood levels of coadministered immunosuppressants will be a complex function of the mutual effects on absorption, distribution, metabolism, and elimination that each drug has on the other. Without knowing how the combined administration of two or more immunosuppressive drugs affects the pharmacokinetics and dynamics of each drug, it is difficult to know how to design in vitro experiments that faithfully reproduce drug interactions in vivo. As a result, there is no guarantee that the drug interactions described in vitro will occur in vivo. Nor is it assured that the results of in vivo studies in one species are predictive how drugs will interact in other species.

Despite this complexity, both in vitro and in vivo studies reached similar general conclusions concerning the effects on the immune system of treatment with RPM plus CsA and RPM plus FK506: these drug combinations produce immunosuppression that is synergistic or additive. RPM and CsA do not antagonize the immunosuppressive effects of each other, and RPM and FK506 are only mutually antagonistic in vitro when one drug is present in substantial molar excess over the other. These findings suggest ways to combine RPM with either CsA or FK506 for optimum clinical immunosuppression bearing in mind the limitations of the information provided by preclinical studies. Finally, combination therapy will only be of practical value if: (1) there is no loss in net immunosuppressive efficacy; (2) overall toxicity is reduced; and (3) excessive immunosuppression is avoided.

Molecular Mechanisms of the Antifungal and Immunosuppressive Activities of RPMs

The field of immunosuppression is entering a new era. Rather than relying on random chance and empiricism, immunosuppressive drug discovery, development, and clinical use is increasingly exploiting more rational approaches that are based on a fundamental understanding of the immune system. For example, an understanding of the cellular and molecular mechanisms of graft rejection coupled with an appreciation of the effects of CsA on immune cells were fundamental to the discovery of FK506 and its subsequent successful use in vivo. Similarly, enough is now known about the unique immunosuppressive and toxic effects of RPM to conduct a rational program to screen for, to develop, and to use new drugs that maximize the desired pharmacological properties of RPM, but that minimize its toxic effects.

However, the odds of discovering improved versions of known immunosuppressants will be increased even more when new molecular structures are actually designed to fulfill specific criteria, rather than identified through rational screening programs. This evolutionary step in the field of immunosuppression depends on the definition of the precise molecular mechanisms by which currently available immunosuppressants affect the immune system and cause toxicity. If the molecular mechanisms of drug action take a long time to define, the speed of rational screening could more than compensate for its relative lack of inefficiency. Neither approach guarantees success because the molecular mechanisms responsible for both efficacy and toxicity may be inseparable.

Any model of the molecular mechanisms of action of RPM will be incomplete unless it can adequately explain: (1) the diverse immunosuppressive effects in vitro and in vivo; (2) the tissue-specific antiproliferative effects; (3) the antifungal and antitumor activities; and (4) the toxicity of RPM. It is also important to note that some of the significant effects of RPM in vivo may be caused not by RPM itself, but by its metabolites. If this is so, molecular mechanisms that revolve solely around the parent compound may not account for all of the effects of RPM in vivo. The interpretation of data that purport to explain the molecular mechanisms of action of RPM is further complicated by the necessarily contrived nature of many of the experimental systems in which highly selected biomolecules, rather than whole cells or

even intact organisms, are used. However, without using highly defined systems to study immunosuppressive drug action, it would be difficult to isolate the molecular targets of the action of those drugs. The limitations of these systems make it imperative that all proposed mechanisms be shown to be operative and nontrivial in whole cells and, even more importantly, in vivo. Unfortunately, our powers of inductive reasoning still leave much to be desired.

Keeping the above cautions in mind, we can review the limited work that attempts to explain the actions of RPM at the molecular level. The molecular mechanisms of the antifungal and immunosuppressive effects of RPM will be discussed because these effects may be related. To date, there have been no studies of the molecular mechanisms of the antitumor activity or toxicity of RPM.

Mechanisms of the Antifungal Activity of RPM

As previously discussed, the antiyeast (*C albicans*) activity from the broth of *S hygroscopicus* led to the isolation and chemical characterization of RPM. Now, a decade and a half later, the effects of RPM on yeast (*Saccharomyces cerevisiae*) are being exploited to uncover its molecular mechanisms of action.[115-117] It has been found that mutant strains of yeast that are resistant to the growth inhibitory effects of RPM lack a functional cytoplasmic protein that binds RPM. This protein is a rotamase enzyme (peptidyl-prolyl isomerase, or PPIase) that catalyzes the interconversion of *cis*- and *trans*-rotamers of peptidyl-prolyl amide bonds in naturally occurring proteins and peptides; both RPM and FK506, but not CsA, bind to and inhibit its enzymatic activity. Expression of the human equivalent of this rotamase (discussed subsequently) restores the sensitivity of yeast to RPM toxicity. FK506 is toxic to yeast without the rotamase, thus indicating that FK506 mediates its effects differently from RPM. Interestingly, CsA is also toxic to yeast and this toxicity is dependent on the presence of a rotamase distinct from the one to which RPM and FK506 bind. Simple inactivation of rotamase activity by RPM cannot be the sole explanation for the toxicity of RPM to yeast for two reasons: (1) mutations in the gene coding for rotamase are nonlethal; and (2) two other yeast gene products are required for RPM to cause toxicity. Therefore, it has been proposed that the interaction among RPM, the rotamase, and other yeast proteins are required for RPM to inhibit yeast growth. Before all the effects of RPM on yeast listed in Table 1 can be explained, much more needs to be learned about the molecular interactions between RPM and its molecular targets in yeast.

Because RPM is a secondary waste metabolite, it is unlikely that it is produced by *S hygroscopicus* for antibiotic purposes. Its binding to the rotamase of yeast and the subsequent toxicity it causes are probably coincidental rather than a result of evolutionary pressures.

Mechanisms of the Immunosuppressive Activity of RPM

Mammalian rotamases are cytosolic or membrane-bound enzymes present in high concentrations that exist as closely related isoforms in two structurally different families: (1) CsA-binding proteins (cyclophilins), and (2) proteins that bind both FK506 (FK506-binding protein, FKBP) and RPM.[44,52,148-160] The mammalian rotamases share a high degree of sequence homology with their yeast counterparts. Although these enzymes have been referred to as immunophilins because of their affinity for the three immunosuppressive drugs, it is important to remember that the normal function of these proteins is probably completely unrelated to their binding of fungal and bacterial natural products. The predominant roles these enzymes play in cellular physiology remain to be determined. Their substrate specificity indicates that they act to accelerate the folding of proteins into functionally active configurations, but the importance of this activity and the reasons for such high cytoplasmic enzyme concentrations are unclear. These rotamases may also play roles in both the trafficking and intracellular translocation of proteins.

Because CsA, FK506, and RPM inhibit rotamase activity in T cells, it was initially thought that this effect was central to the suppression of T-cell proliferation by these drugs.[161-163] Just as the inhibition of yeast rotamase activity is insufficient to explain the effects of RPM in that organism, the following evidence also indicates that suppression of T-cell activation by these drugs involves more than their inhibition of T-cell rotamase activity: (1) CsA and FK506 analogues that bind to and inhibit the enzymatic activity of their respective rotamases are not immunosuppressive[164-165]; (2) RPM binds to and inhibits the same rotamase as FK506,[141] but has distinctly different effects on the immune system in vivo and in vitro compared with FK506; and (3) the 50% inhibitory concentration (IC_{50}) for suppression of T-cell

function in vitro by FK506 is about 10-fold less than the cytoplasmic concentration of FKBP.[29,110]

New data from cell-free systems suggests an alternative explanation for the suppression of the Ca^{2+}-dependent signal transduction pathway in T cells by CsA-cyclophilin or FK506-FKBP complexes[155-157] (Fig 9). Both of these drug-rotamase complexes bind the Ca^{2+}-calmodulin–dependent serine/threonine phosphatase, calcineurin. This interaction inhibits the serine/thereonine phosphatase activity of calcineurin as well as alters its substrate specificity. Furthermore, it is now known that CsA and FK506 inhibit the Ca^{2+}-dependent translocation of the cytosolic component of a transcription factor (NF-AT) to the nucleus where it combines with its nuclear component to enable the transcription of early phase T-cell activation genes. Although there is no direct proof, it has been suggested that these drug-rotamase complexes may act by inhibiting the putative calcineurin-mediated dephosphorylation of the cytoplasmic component of NF-AT that may be required for the translocation of this factor into the nucleus.[155,156,166] It has not been demonstrated whether the drug-rotamase complexes formed in the presence of immunosuppressive drug concentrations have the same effects on calcineurin in T cells in vitro and in vivo as they do in cell-free systems or whether there is a direct causal relationship between this effect and the suppression of Ca^{2+}-dependent T-cell activation in general, and NF-AT translocation in particular. In any case, the discovery that a CsA analogue that inhibits T-cell proliferation in vitro as well as its CsA parent, but binds to cyclophilin 100-times less actively than CsA,[165] needs to be reconciled with molecular mechanisms of immunosuppressant drug action that depend on the formation drug-rotamase complexes.

As previously mentioned, RPM binds to (RPM dissociation constant K_d = 0.2 nm, FK506 K_d = 0.4 nm) and inhibits (RPM inhibition constant K_i = 0.2 nm, FK506 K_i = 1.7 nm) the enzymatic activity of the predominant intracellular rotamase receptor for FK506, an FKBP of relative molecular mass (M_r) 11,819 (FKBP-12).[116,153,154,167] The solution structure of FKBP-12 by NMR spectroscopy shows the rotamase-active site to be a pocket comprised of aromatic amino acids suited for the interaction with proline rings in natural peptide substrates. The structure of 13-kd and 27-kd FK506- and RPM-binding proteins with high amino sequence identity to FKPB-12 shows that the aromatic residues that line the hydrophobic pockets are conserved regardless of the isoform of FKBP.[151] X-ray crystallographic data on complexes between FKBP and FK506 and between FKBP and RPM show that FK506 accommodates to binding by FKBP by altering its amide bond from the *cis* to *trans* form.[168] The bound form of FK506 resembles unbound RPM, thus providing an explanation for the higher affinity of binding of RPM (Fig 10). These data also support the suggestion that FK506 and RPM bind to FKBP as transition-state analogues of a leucyl-(twisted amide-)prolyl peptide substrate for FKBP.[44,162-164] The identical structural segments of both FK506 and RPM that bind to FKBP have been referred to as the "binding domains" of these macrolides; the remainder of both molecules differ structually from one another. Because FK506 and RPM have different effects on immune cells, it was suggested that these areas ("effector domains") are responsible for these biological differences. This is a very useful conceptual construct[44,161] that can be used to relate the structure of members of this class of macrolides to their activity.

As previously noted, RPM inhibits the growth of yeast by binding to yeast FKBP or even human FKBP expressed in yeast. For the RPM-FKBP complex to mediate its antifungal effect, the conformation of the effector domain may be critical. Because 29-demethoxyrapamycin is a far less potent antiyeast drug than RPM in vitro and in vivo (discussed in sections headed In Vitro Antimicrobial Activity of RPM and Effects of RPM on *Candida* Infections In Vivo), C29 may be part of the antifungal effector domain of RPM. Our recent finding that 29-demethoxyrapamycin prolongs graft survival far less potently and effectively than RPM (Morris RE: unpublished observations, 1991) suggests that similar effector domains are required for both the antifungal and the immunosuppressive activities of RPM. The fact that the acute toxicity of 29-demethoxyrapamycin is less than RPM indicates that the structure of RPM at the C29 position participates in reactions that cause adverse effects. Alternatively, 29-de-

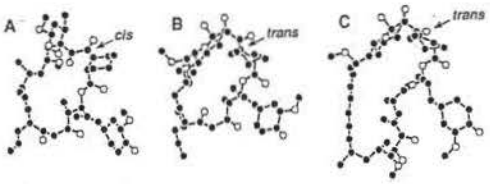

Figure 10. Structures of FK506 and RPM from x-ray diffraction studies. (A) Unbound FK506. (B) FK506 bound to FKBP. (C) Unbound RPM.[168]

methoxyrapamycin may be less toxic and less effective than RPM because 29-demethoxyrapamycin is less stable or less bioavailable.

Several lines of evidence support the contention that RPM-FKBP complexes are necessary for RPM to suppress T-cell proliferation. An FK506 analogue, 506BD, is devoid of the effector domain causing it to be nonimmunosuppressive, but because it retains the binding domain, it binds to FKBP normally.[41,164] The inhibition by RPM of IL-2–mediated CTLL proliferation is blocked by 506BD. Many other suppressive effects of RPM on T cells are antagonized by high concentrations of FK506 that compete with RPM for binding to FKBP (discussed in section headed RPM-Immunosuppressive Drug Interaction). Unlike the FK506-FKBP complex, the effector domain of the RPM-FKBP complex does not bind to calcineurin/calmodulin nor does it inhibit the translocation of the cytoplasmic component of NF-AT into the nucleus after T-cell activation.[152-166] These differences between the actions of FK506-FKBP complexes and RPM-FKBP complexes are consistent with the known differences in the sites of action of these two drugs in the biochemical pathways leading to T- and B-cell proliferation.

Although there is no direct experimental data that suggest the exact molecular mechanisms by which the RPM-FKBP complex inhibits both Ca^{2+}-dependent and -independent T- and B-cell proliferation, all available evidence indicates that RPM prevents T-cell proliferation by acting in G_1 to prevent the transition to the S phase of the cell cycle (discussed in section headed Effects of RPM on Immune Cells in Vitro). Studies from nonimmune cells have shown that growth factors commonly transmit signals from the cell membrane to the nucleus through successive cytoplasmic reactions that ultimately result in DNA proliferation.[169] Before cells can respond to signal transduction that leads to DNA synthesis they must be "competent." T cells become "competent" as a result of events in the transition from G_0 to G_1 caused by engagement of the TCR/CD3 complex with antigen (signal 1) and by costimulatory signals (signal 2). The binding of a critical threshold of IL-2 to high-affinity IL-2 receptors provides the quantal signal needed for T cells to commit to DNA synthesis and mitosis.[129,170]

The precise sequence of biochemical reactions that transduce the IL-2 signal from the IL-2 receptor to nuclear elements that initiate DNA synthesis is unknown. New information from studies of IL-2 receptor signal transduction as well as studies of molecules that participate in the G_1 to S transition in nonimmune cells and T cells has contributed to our understanding of cell cycle regulation at the molecular level (Fig 9). Protein phosphorylation and dephosphorylation are "on-off" switches that are tripped as part of the progression through G_1 in many mammalian cells.[171-174] Therefore, it was not surprising to learn that the transduction of the signal from the interaction of IL-2 with its receptor is caused by protein tyrosine kinase activity directly coupled to the IL-2R resulting in the phosphorylation of the receptor and other cellular substrates.[130] In nonimmune cells regulation of the cell cycle has been shown to be affected by a product of the *cdc2* gene ($p34^{cdc2}$), a serine/threonine kinase, that is activated by another group of proteins known as cyclins. Together, the complex between the *cdc2* kinase and cyclin form a "maturation promoting factor" that seems to act late in G_1 at the mammalian equivalent of the "start" point in yeast to commit cells to DNA synthesis.[175-178] Although T cells in G_0 have low levels of *cdc2* messenger RNA and *cdc2* kinase, RNA and protein levels increase during late G_1 or early S phase after mitogenic activation of T cells.[179] An antisense oligodeoxynucleotide complementary to the *cdc2* mRNA reduced the entry of T cells into S phase but had no effect on early G_1 events including the expression of the IL-2 and transferrin receptors or proteins induced by *c-myc* or *c-myb*. The *cdc2* kinase may participate in the phosphorylation of the retinoblastoma gene product, Rb, because activated T cells treated with antisense *cdc2* oligomers reduced the amount of Rb-phos normally present in G_1-S. The state of Rb phosphorylation may play a major role in the regulation of the cell cycle because transcription factors bound to Rb dissociate from Rb-phos and are then free to enable cells to exit from G_1.[180]

Metcalf and Milner were the first to examine the role of phosphatases as inhibitors of T-cell activation by immunosuppressants.[137] They showed that okadaic acid, an inhibitor of the serine/threonine phosphatases PP1 and PP2A, does not block FK506-sensitive steps in the transition from G_0 to G_1, but does inhibit mitogenesis in late G_1. Two substrates for which dephosphorylation by phosphatases may be required for G_1 to S progression were suggested: the *jun* component of the *jun-fos* heterodimeric transcription factor, and *c-myb*.[179] These investigators also showed that both RPM and okadaic acid block mitogen-induced T-cell proliferation at similar points in G_1[126] suggesting that RPM might act by inhibiting PP1 and PP2A phosphatases. Although pure RPM

does not inhibit either the activity of PP1 or PP2A, the RPM-FKBP complex may. Alternatively, the RPM-FKBP complex may directly or indirectly prevent the phosphorylation of Rb or other proteins or may interfere with transcription factors that are required to initiate DNA synthesis. Just as FK506 and CsA are being used to elucidate the mechanics of signal transduction after the binding of ligands to the TCR/CD3 complex and accessory molecules, RPM will be a useful tool to dissect the events subsequent to the bindng of IL-2 to its receptor that led to commitment to T-cell DNA synthesis. RPM may also have general application for the study of the intracellular events triggered by the binding of a wide variety of cytokine and growth factor ligands with their receptors. The results from these studies should help explain why the inhibition of cell proliferation by RPM is relatively selective, and why it fails to inhibit the multiplication of cells in the bone marrow and other cells that depend on growth factors.

RPM may be a prototype of a new class of drugs that inhibit growth factor-mediated events. If this could be shown in additional investigations, it would explain how RPM successfully prevents and reverses acute allograft rejection as well as prevents chronic graft vessel disease. Inhibition of growth factors may even help explain the dramatic effects RPM has on intrathymic T-cell differentiation. Ultimately, RPM may find therapeutic application outside the immune system as a means to inhibit undesired growth factor effects for the treatment of cancer and many other disorders caused by growth factor-mediated cell proliferation.

Finally, the molecular mechanisms for suppression of T- and B-cell proliferation proposed from studies of cell-free and whole cell systems in vitro will have to be verified in vivo. For example, the effects of CsA treatment on events leading to activation of immune cells stimulated by alloantigen in vivo have been found to be quite different from the effects of CsA in vitro.[101]

Conclusion

As we scan the knowledge of RPM that has accumulated over the last 15 years, it is easy to see islands of superficial clarity separated by oceans of ignorance. A complete understanding of RPM would enable us to explain the antifungal, antitumor, antiproliferative, immunosuppressive, and toxic effects of RPM at the most fundamental molecular level.

The ease of creating mutations in yeast suggests that the molecular mechanisms by which RPM suppresses the growth of this organism will yield to investigation before we completely understand the effects of RPM on mammalian cells. We already know that RPM must be bound by the yeast equivalent of FKBP, and that at least two other gene products are required for RPM to inhibit yeast growth. Because the cell cycles of yeast and of mammalian cells are regulated in similar ways, the elucidation of the effects of RPM on yeast should enhance our ability to define its actions in mammalian cells. Most important will be to understand how the effector domain of RPM interacts with yeast molecules. On a more practical note, it is not yet known whether the anti-*C albicans* activity of RPM will be of any therapeutic value. The immunosuppressive activity of RPM eliminates this as a primary indication for its use, but if, in patients requiring immunosuppression, RPM blood levels that provide the optimum balance between immunosuppressiuve efficacy and toxicity also are high enough to provide antifungal activity, it would be a welcome side benefit. Because RPM will be used initially with other immunosuppressants for patients in whom the risk of overimmunosuppression is a concern, the antifungal activity of RPM may be of secondary importance.

In addition to its potent and effective actions on immune cells, it is important to note that RPM is also able to suppress the ongoing proliferation of nonlymphoid tumor cells; in vitro RPM also halts the proliferation of transformed T and B cells that are resistant to the antiproliferative effects of CsA and FK506. Because RPM does not act as a completely nonspecific antiproliferative agent in vivo, it will be interesting to see how restricted its antiproliferative effects are when cells of different origins are exposed to RPM. The antiproliferative effects of RPM may even be exploited for the treatment of a wide variety of diseases caused by cell division that is inappropriate in time or place. Finally, an understanding of the molecular mechanisms by which RPM halts the cycling of constitutively dividing cells may not only shed light on tumorigenesis, but also tell us more about how the mammalian cell cycle is regulated. Is the efficacy of the antiproliferative activity of RPM related to the dependence of a cell on growth factors? Does RPM act via a complex with intracellular binding proteins, and if so, what is the nature of this interaction? The minimal antitumor activity of 29-demethoxyrapamycin suggests that the structure at the C29 position is just as important for the antipro-

liferative activity of RPM as it is for its antifungal and immunosuppressive activities.

Because RPM has the potential for toxicity, it will be evaluated as an immunosuppressant in organ allograft patients before its use is seriously contemplated in patients with non–life-threatening chronic autoimmune disease. Using the transplant patient as an example, we can try to imagine shrinking to enter the molecular world, grasping onto the macrolide ring of RPM to get a first hand view of how RPM travels throughout the tissues of recipients treated with this drug. After IV administration, the highly lipophilic RPM is immediately bound by serum proteins and the unbound RPM passes easily through cell membranes and, through its binding domain, forms tight complexes in the pockets of cytoplasmic FKBPs. Because FKBPs are widely distributed, RPM also is widely distributed throughout most tissues. Unless the concentrations of RPM are kept to a minimum, RPM may cause toxicity to the brain, kidneys, gastrointestinal tract, testicles, and islets. Because RPM initially will be used as adjunctive treatment in patients receiving conventional immunosuppression, the possibility of overlapping toxicity among immunosuppressants will exist. However, if our experience in the monkey is predictive of the use of RPM in humans, the use of both RPM and CsA at low doses may provide the most effective immunosuppression with the least toxicity. After the completion of phase I trials, we will know the dose-limiting toxicities of RPM, and then in vitro and in vivo models can be used to dissect the mechanisms by which RPM causes these untoward effects. Using this information, second-generation agents with less toxicity may be able to be designed. In the meantime, careful blood level monitoring of RPM may enable the concentrations of RPM in all tissues to be kept low, but high enough in immune cells to produce the immunosuppression required. The relationship between RPM blood levels and tissue levels will need to be validated.

Our view of the molecular actions of RPM is enhanced by results from experiments that compare the effects of RPM and 29-demethoxyrapamycin. Because the acute toxicity of 29-demethoxyrapamycin is much less than RPM, the O-methyl group at the C29 position may contribute to the molecular actions that lead to toxicity. The conformation in the region of RPM comprising the C29 position may affect the antifungal, antitumor, immunosuppressive, and toxic activities of RPM: (1) by altering the stability, bioavailability, or metabolism of RPM; (2) because this region of the molecule acts through a common biochemical pathway to produce all of the biological effects of RPM or, (3) because this substructure interacts with different pathways each of which is responsible for the diverse actions of RPM. If trivial explanations for the pharmacological differences between RPM and 29-demethoxyrapamycin that are based on differences in the stability, absorption, distribution, metabolism, and elimination of these RPM are ruled out, we can conclude that the C29 position of RPM is an important pharmacophore. Because the C29 position is not part of the binding domain, 29-demethoxyrapamycin, like 506BD, should bind well to FKBP and should not account for the differences in the activities between RPM and 29-demethoxyrapamycin. Because the C29 position is part of the putative effector domain of RPM, the presence or absence of the O-methoxy group in this position may be critical to the interactions of the effector domain with molecules that mediate the biological effects of RPM. We are now testing this hypothesis experimentally to form a more complete understanding of the structure-function relationships of RPM.

Because 29-demethoxyrapamycin is less toxic, but also a less potent drug, only a careful comparison between the therapeutic indexes of RPM and 29-demethoxyrapamycin can determine which is the preferred form. Even if 29-demethoxyrapamycin is not found to be superior to RPM, other structural modifications in the effector domain of RPM may produce the desired properties. This strategy assumes that the molecular mechanisms responsible for the therapeutic effects of RPM are not identical to the mechanisms that cause toxicity. As these molecular interactions become better understood, the validity of this assumption and the strategy for analogue development will become clearer.

As we continue our journey with RPM in vivo, we can try to image how RPM causes its immunosuppressive effects. For example, after RPM enters resting and activated T and B cells, it will be confronted with an excess of FKBP isoforms and maybe other molecules that have yet to be characterized to which RPM binds avidly through its binding domain leaving its effector domain exposed. Because of either its low dissociation from its FKBPs or its unknown and slowly reversible effects, RPM is immunosuppressive even when administered infrequently. The CsA in the cell is bound to its cyclophilins and, as a result, prevents the transcription of early phase T-cell activation genes when T cells are activated by alloantigen.

If transcription does occur because CsA is incompletely effective or because active rejection is ongoing, immune cells, graft endothelium, and other cells in the graft will be bathed in high concentrations of cytokines and growth factors. If RPM acts to block the signal transduction of cytokines through Ca^{2+}-independent pathways, it could be a valuable complement to CsA because RPM will prevent IL-2–induced proliferation of T cells. RPM should also blunt the effects of cytokines on other cells (eg, upregulation of cell surface receptors, alloantigens, and adhesion molecules). RPM in B cells is presumably complexed to FKBPs and prevents B-cell proliferation and synthesis of immunoglobulin. All these actions (and many more yet to be discovered) help explain how RPM can prevent and treat acute and accelerated rejection, and how it can prevent the obliterative vasculopathy (a growth factor-mediated disease) that is associated with chronic rejection. Yet to be defined is exactly how the effector domain of RPM as part of FKBP-RPM complexes prevents growth factor-independent cell proliferation, or how the effector domain interrupts the pathways used by cytokines and growth factors to transduce their signals to the nucleus; testable hypotheses were advanced in this review. It is also not clear why the effects of RPM are more profound on immune cells than on other cell types. How can RPM block the effects of certain growth factors and not others? Maturation of cells in the bone marrow is highly dependent on growth factors, yet there is little evidence that RPM suppresses the bone marrow.

In mice and rats, RPM also enters thymocytes and stromal cells and causes thymocyte depletion that is reversible after cessation of RPM treatment. As previously discussed, this effect may facilitate the induction of donor-specific unresponsiveness via clonal deletion as immature thymocytes are exposed to donor antigen when the thymus recovers from the effects of RPM. There are other mechanisms that may explain how treatment with RPM promotes indefinite graft survival. Because RPM also blocks costimulatory signals in cells that are receptive to signals from the TCR/CD3 complex, RPM may facilitate the induction of peripheral anergy. A better understanding of the "tolerogenic" effects if RPM in the rodent should help untangle the complexities of thymocyte maturation and events leading to "self-nonself" discrimination. Ultimately, this knowledge may help us devise ways of inducing tolerance in large animals. Perhaps RPM combined with other immunosuppressive regimens and donor bone marrow will promote the induction of donor-specific unresponsiveness in large animals and humans.

Even though many of these same arguments can be used to explain how RPM treatment modifies autoimmune disease in experimental animals, it is important to remember that only a few of the effects of RPM on immune cells in vitro have been evaluated in vivo. Before considerable effort is expended defining the precise mechanisms of action of RPM on immune cells in vitro, it might be wise to insure that RPM has similar effects on cells in vivo.

For better or worse, immunosuppression has entered an era dominated by xenobiotic fermentation products. This is partly a legacy of CsA, but the field now has a momentum of its own and will be perpetuated for several years. This era has produced a bumper crop of new drugs and this success should provide the critical mass for even more rapid growth in the future. Fortunately, these xenobiotics and new synthetic compounds block the immune response at different points (Fig 3). Therefore, these molecules can be used creatively to study specific segments of immune activation pathways so that the biochemistry of signal transduction is defined more clearly. Of more immediate importance, these new molecules enable combination immunosuppressive therapy to reach new levels of sophistication. These possibilities offer the hope that rejection can be prevented and treated more effectively and safely than ever before.

Nevertheless, we should remember that all these immunosuppressants regulate immune cells by means that are probably very different from the way immune cells regulate themselves. When we can exploit the immunoregulatory circuits of our own body we will be able to control the immune reactions even more effectively and safely; then we will finally be working with, rather than against, the immune system.

Acknowledgment

I acknowledge the able assistance of the following members of my laboratory without whose dedicated effort our studies of RPM could not have been completed: Drs J. Wang, B. Zheng, and B.M. Meiser, and Mrs R. Shorthouse and Mr J. Wu. I was fortunate to be given the opportunity to study RPM by Dr S.N. Sehgal of Wyeth-Ayerst Pharmaceuticals. I also wish to thank those from my laboratory (Mr E. Nakakura, Dr B. Zheng, Mr M. Murphy, Mr J. Wu and Dr C. Gregory) and from elsewhere (Dr S.N. Sehgal of Wyeth-Ayerst, and Drs J.R. Luly and J.B. McAlpine of Abbott Laboratories) for their critical comments on the manuscript. Finally, I will always be grateful for Dr N.L. Tilney's infinite reservoir of patience and good humor during the long and much

overdue gestational process that led to the ultimate birth of this review.

Appendix

The quantal form of heart bioassay was used to determine immunosuppressive drug potency (ED_{50} = effective dose giving 50% hearts beating on day 14), relative drug potency (potency ratio), and immunosuppressive drug-drug interaction. For each treatment, λ, we obtained estimates of the $(ED_{50})_\lambda$, $(\widehat{ED}_{50})_\lambda$, and the slope, $\hat{\beta}_\lambda$ by performing a logistic regression analysis of percent heart graft survival at day 14 on log dose. The estimated probability of hearts beating at day 14 when using treatment λ and dose D is then given by:

$$\frac{1}{1 + \exp(\hat{\beta}_\lambda \cdot \log((\widehat{ED}_{50})_\lambda - \hat{\beta}_\lambda \cdot \log(D))}.$$

We tested whether the log dose-response curves of two treatments, treatment_1 and treatment_2, were parallel on the logit scale by testing whether $|\beta_1 - \beta_2| > 0$. When this test did not show a difference in slopes between the two curves, we estimated the log relative immunosuppressive drug potency to be the horizontal differences between the two dose-response curves on the logit scale, ie,

$$\hat{\beta} \cdot [\log((\widehat{ED}_{50})_1) - \log((ED_{50})_2)]$$

where $\hat{\beta}$, the estimated common slope, $(\widehat{ED}_{50})_1$, and $(\widehat{ED}_{50})_2$ are obtained by fitting:

$$\frac{1}{1 + \exp(\beta \cdot [\delta_{i1} \log((ED_{50})_1) + \delta_{i2} \log((ED_{50})_2)] - \beta \cdot \log(D))}, \quad \delta_{ij} = \begin{cases} 1 & i = j, \\ 0 & i \neq j \end{cases}$$

to the combined data from treatments 1 and 2.[102,481]

References

1. Morris RE, Meiser BM: Identification of a new pharmacologic action for an old compound. Med Sci Res 1989, 17:609
2. Calne RY, Collier DS, Lim S, et al: Rapamycin for immunosuppression in organ allografting. Lancet 1989, 2:227
3. Meiser BM, Wang J, Morris RE: Rapamycin: A new and highly active immunosuppressive macrolide with an efficacy superior to cyclosporine, in F. Melchers (ed): Progress in Immunology, Proceedings of the 7th International Congress of Immunology. Berlin, Germany, Springer-Verlag, 1989, p 1195
4. Morris RE, Wu J, Shorthouse R: A study of the contrasting effects of cyclosporine, FK 506, and rapamycin on the suppression of allograft rejection. Transplant Proc 1990, 22:1638
5. Morris RE, Wang J, Wu J, et al: Induction of graft tolerance by brief treatment with rapamycin (RPM): A new, far more potent and effective immunosuppressant than cyclosporine (CsA). J Heart Transplant 1990, 9:62
6. Morris RE, Meiser BM, Wu J, et al: Use of rapamycin for the suppression of alloimmune reactions in vivo: Schedule-dependence, tolerance induction, synergy with cyclosporine and FK506 and effect on host-versus-graft and graft-versus-host reactions. Transplant Proc 1991, 23:521
7. Morris RE: Rapamycin: FK506's fraternal twin or distant cousin? Immunol Today 1991, 12:137
8. Morris R, Wang J, Gregory C, et al: Initial studies of the efficacy and safety of rapamycin (RPM) administered to cynomolgus monkey recipients of heart allografts. J Heart Lung Transplant 1991, 10:182
9. Morris RE: In vivo immunopharmacology of the macrolides FK506 and rapamycin: Toward the era of rational immunosuppressive drug discovery development and use. Transplant Proc 1991, 23:2722
10. Meiser BM, Billingham ME, Morris RE: The effect of cyclosporine and two new immunosuppressive macrolides, FK506 and rapamycin, on heart graft rejection and graft coronary atherosclerosis. J Heart Transplant 1990, 9:55
11. Meiser BM, Billingham ME, Morris RE: Graft vessel disease: The role of rejection and the effect of cyclosporine, FK506, and rapamycin. Lancet 1991, 338:1297
12. Morris RE, Hoyt EG, Eugui EM, et al: Prolongation of rat heart allograft survival by RS-61443. Surg Forum 1989, 40:337
13. Morris RE, Hoyt EG, Murphy MP, et al: Mycophenolic acid morpholinoethyl ester (RS-61443) is a new immunosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. Transplant Proc 1990, 22:1659
14. Morris RE, Hoyt EG, Wang J: RS-61443 (RS) is a novel and specific inhibitor of T and B cell purine synthesis that induces transplant tolerance, reverses acute rejection and prolongs xenograft survival. J Heart Transplant 1990, 9:62
15. Morris RE, Wang J, Blum JR, et al: Immunosuppressive effects of the morpholinoethyl ester of mycophenolic acid (RS-61443) in rat and nonhuman primate recipients of heart allografts. Transplantation Proceedings, 1991, 23:(2, Suppl 2): 19-25
16. Morris RE, Wang J: Comparison of the immunosuppressive effects of mycophenolic acid and the morpholinoethyl ester of mycophenolic acid (RS-61443) in recipients of heart allografts. Transplant Proc 1991, 23:493
17. Morris RE, Hoyt EG, Murphy MP, et al: Immunopharmacology of FK-506. Transplant Proc 1989, 21:1042
18. Morris RE, Wu J, Shorthouse R: Comparative immunopharmacologic effects of FK 506 and CyA in in vivo models of organ transplantation. Transplant Proc 1990, 22:110
19. Flavin T, Ivens K, Wang J, et al: Initial experience with FK506 as an immunosuppressant for nonhuman primate recipients of cardiac allografts. Transplant Proc 1991, 23:531
20. Yuh DD, Morris RE: 15-Deoxyspergualin is a more potent and effective immunosuppressant than cyclosporine but does not effectively suppress lymphoproliferation in vivo. Transplant Proc 1991, 23:535
21. Morris RE: 15-Deoxyspergualin: Amystery wrapped in an enigma Clin Traansplant 1991, 5:530
22. Murphy MP, Morris RE: The selective antimetabolite brequinar sodium potently and effectively suppresses heart allograft rejection. Med Sci Res (in press)
23. Ryffel B: Pharmacology of cyclosporine VI. Cellular activation: Regulation of intracellular events by cyclosporine. Pharmacol Rev 1989, 41:407
24. Kay JE, Moore A, Doe SEA, et al: The mechanism of action of FK 506. Transplant Proc 1990, 22:96

25. Lin CS, Boltz RC, Siekierka JJ, et al: FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes. Cell Immunol 1991, 133:269
26. Staruch MJ, Melino M, Koprak S, et al: FK506 and rapamycin inhibit murine T cell activation through different mechanisms. FASEB J 1989, 3:3411 (abstr A813)
27. Tocci MJ, Matkovich DA, Collier KA, et al: The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J Immunol 1989, 143:718
28. Metcalfe SM, Richards FM: Cyclosporine, FK506, and rapamycin. Some effects on early activation events in serum-free, mitogen-stimulated mouse spleen cells. Transplantation 1990, 49:798
29. Dumont FJ, Staruch MJ, Koprak SL, et al: Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J Immunol 1990, 144:251
30. Kay JE, Kromwel L, Doe SE, et al: Inhibition of T and B lymphocyte proliferation by rapamycin. Immunology 1991, 72:544
31. Sigal NH, Lin CS, Siekierka JJ: Inhibition of human T-cell activation by FK 506, rapamycin, and cyclosporine A. Transplant Proc 1991, 23:1
32. Turka LA, Dayton J, Sinclair G, et al: Guanine ribonucleotide depletion inhibits T cell activation. J Clin Invest 1991, 87:940
33. Allison AC, Almquist SJ, Muller CD, et al: *In vitro* immunosuppressive effects of myclophenolic acid and an ester pro-drug, RS-61443. Transplant Proc 1991, 23:10 (suppl 2)
34. Peters GJ, Schwartsmann G, Nadal JC, et al: In vivo inhibition of the pyrimidine de novo enzyme dihydroorotic acid dehydrogenase by brequinar sodium (DUP-785; NSC 368390) in mice and patients. Cancer Res 1990, 50:4644
35. Luly JR: Mechanism-Based Immunosuppressant: in Bristol JA (ed): Annual Reports in Medicinal Chemistry. Academic Press, San Diego, CA, 1991, 26:211
36. Devlin JP, Hargrave KD: Tetrahedron report number 256: The design and synthesis of immune regulatory agents: Targets and approaches. Tetrahedron 1989, 45:4327
37. Caufield CE, Musser JH: Macrocyclic Immunomodulators: in Bristol JA (ed): Annual Reports in Medicinal Chemistry. Academic Press, San Diego, CA, 1990, 25:195
38. Collier SJ: Immunosuppressive drugs. Curr Opin Immunol 1990, 2:854
39. Sehgal SN, Chang JY: Rapamycin: A new immunosuppressive macrolide. Transplant Immunol Lett 1990, 7:12
40. Chang JY, Sehgal SN: Pharmacology of rapamycin: A new immunosuppressive agent. Br J Rheumatol 1991, 30:62 (suppl 2)
41. Chang JY, Sehgal SN, Bansbach CC: FK506 and rapamycin: Novel pharmacological probes of the immune response. Trends Pharmacol Sci 1991, 12:218
42. Kahan BD, Chang JY, Sehgal SN: Preclinical evaluation of a new potent immunosuppressive agent, rapamycin. Transplantation 1991, 52:185
43. Sigal NH, Siekierka JJ, Dumont FJ: Observations on the mechanism of action of FK-506: A pharmacologic probe of lymphocyte signal transduction. Biochem Pharmacol 1990, 40:2201
44. Schreiber SL: Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 1991, 251:283
45. Nossal GVJ: Summary of the first international FK506 congress: Perspectives and prospects. Transplant Proc 1991 (in press)
46. Vezina C, Kudelski A, Sehgal SN: Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. Antibiot (Tokyo) 1975, 28:721
47. Sehgal SN, Baker H, Vezina C: Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J Antibiot (Tokyo) 1975, 28:727
48. Baker H, Sidorowicz A, Sehgal SN, et al: Rapamycin (AY-22,989), a new antifungal antibiotic. III. In vitro and in vivo evaluation. J Antibiot (Tokyo) 1978, 31:539
49. Martel RR, Klicius J, Galet S: Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J Physiol Pharmacol 1977, 55:48
50. Findlay JA, Radics L: On the chemistry and high field nuclear magnetic resonance spectroscopy of rapamycin. Can J Chem 1980, 58:579
51. Douros J, Suffness M: New antitumor substances of natural origin. Cancer Treat Rev 1981, 8:63
52. Harding MW, Galat A, Uehling DE, et al: A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature 1989, 341:758
53. Goto T, Kino T, Hatanaka H, et al: Discovery of FK-506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*. Transplant Proc 1987, 19:4 (suppl 6)
54. Tanaka H, Kuroda A, Marusawa H, et al: Structure of FK506: A novel immunosuppressant isolated from streptomyces. J Am Chem Soc 1987, 109:5031
55. Babny G, Morris RE, Babny I, et al: Evaluation of the in vivo dose-response relationships of immunosuppressive drugs using a mouse heart transplant model: Application to cyclosporine. J Pharmacol Exp Ther 1988, 244:259
56. Sehgal SN, Baker H, Eng CP, et al: Demethoxyrapamycin (AY-24,668), a new antifungal antibiotic. J Antibiot (Tokyo) 1983, 36:351
57. Pavia NL, Demain AL: Incorporation of acetate, propionate, and methionine into rapamycin by *Streptomyces hygroscopicus*. J Nat Prod 1991, 54:167
58. Singh K, Sun S, Vezina C: Rapamycin (AY-22,989), a new antifungal antibiotic. IV. Mechanism of action. J Antibiot (Tokyo) 1979, 32:630
59. Swindells NDC, White PS, Findlay JA: The x-ray crystal structure of rapamycin, $C_{51}H_{79}NO_{13}$. Can J Chem 1978, 56:2491
60. McAlpine JB, Swanson SJ, Jackson M, et al: Revised NMR assignments for rapamycin. J Antibiot (Tokyo) 1991, 44:688
61. White PS, Swindells DCN: The structure of the antifungal antibiotic rapamycin. Acta Crystallogr A 1981, 37:C75
62. Paiva NL, Demain AL: Incorporation of acetate, propionate, and methionine into rapamycin by *Streptomyces hygroscopicus*. J Nat Prod 1991, 54:167
63. Findlay JA, Liu J-S, Burnell DJ: The structure of demethoxyrapamycin. Can J Chem 1982, 60:2046
64. Stella VJ, Kennedy PE: Prodrugs of rapamycin. US Patent no. 4,650,803, Mar 17, 1987
65. Supko JG, Malspeis L: Dose-dependent pharmacokinetics of rapamycin prodrug (NSC 506698). Proc Am Assoc Cancer Res 1990, 31:382
66. Napoli KL, Kahan BD: High-performance liquid chromatography of rapamycin. Clin Chem 1991, 37:294
67. Venkataramanan R, Jain A, Cadoff E, et al: Pharmacokinet-

ics of FK 506: Preclinical and clinical studies. Transplant Proc 1990, 22:52
68. Whiting PH, Woo J, Adam BJ, et al: Toxicity of rapamycin—A comparative and combination study with cyclosporine at immunotherapeutic dosage in the rat. Transplantation 1991, 52:203
69. Collier DS, Calne R, Thiru S, et al: Rapamycin in experimental renal allografts in dogs and pigs. Transplant Proc 1990, 22:1674
70. Tiebosch A, Ericzon BG, Wijnen R, et al: Side effects of FK 506 in cynomolgus monkeys: A histopathologic study. Transplant Proc 1990, 22:1652
71. Wijnen RMH, Ericzon BG, Tiebosch ATGM, et al: Noncorrelation of FK506 serum levels and toxicity in the nonhuman primate. Transplant Proc (in press)
72. Collier DSJ, Calne RY, Pollard SG, et al: Rapamycin in experimental renal allografts in primates. Transplant Proc 1991, 23:2246
73. DiJoseph JF, Chang JY: Effect of rapamycin on kidney function in the sprague-dawley rat. FASEB J 1991, 5:1207 (abstr)
74. DiJoseph JF, Chang JY: Effect of rapamycin on the sprague-dawley rat. Transplantation (in press)
75. Eng CP, Sehgal SN, Vezina C: Activity of rapamycin (AY-22,989) against transplanted tumors. J. Antibiot (Tokyo) 1984, 37:1231
76. Houchens DP, Ovejera AA, Riblet SM, et al: Human brain tumor xenografts in nude mice as a chemotherapy model. Eur J Cancer Clin Oncol 1983, 19:799
77. Borel JF: The history of cyclosporin a and its significance in White DJG (ed): Cycloporin A. Elsevier, Amsterdam, The Netherlands, 1982, pp 5-17
78. Di Padova FE: Pharmacology of cyclosporine. V. Pharmacological studies on immune function: In vitro studies. Pharmacol Rev 1989, 41:373
79. Wu J, Palladino MA, Figari IS, et al: Comparative immunoregulatory effects of rapamycin, FK506 and cyclosporine on mitogen-induced cytokine production and lymphoproliferation. Transplant Proc 1991, 23:238
80. Adams LM, Caccese R, Cummons T, et al: Effect of the immunosuppressants rapamycin and cyclosporin A on collagen-induced arthritis in mice. FASEB J 1990. 4:358 (abstr)
81. Carlson R, Calhoun W, Lugay J, et al: Oral effect of rapamycin on T-cell mediated inflammation in rodent models. FASEB J 1990, 4:1021 (abstr)
82. Warner LM, Cummons TA, Chang JY, et al: Comparison of rapamycin and cyclosporine A effects on murine systemic lupus erythematosus. FASEB J 1990, 4:358 (abstr)
83. Adams LM, Caccese R, Cummons T, et al: Effect of the immunosuppressants rapamycin (RAP) and cyclosporin A (CsA) on collagen-induced arthritis (CIA) in mice FASEB J 1990, 4:358 (abstr)
84. De Paulis A, Cirillo R, Ciccarelli A, et al: FK-506, a potent novel inhibitor of the release of proinflammatory mediators from human FcŒERI + cells. J Immunol 1991, 146:2374
85. Hultsch T, Schreiber SL, Hohman RJ: Inhibition of IgE receptor mediated exocytosis from rat basophilic leukemia cells by FK-506 is reversed by rapamycin: Evidence for common signaling pathways in mast cells and T-lymphocytes. FASEB J 1991, 5:1008 (abstr)
86. Morris RE, Meiser BM: Identification of a new pharmacologic action for an old compound. Med Sci Res 1989, 17:877
87. Wang J, Morris RE: Effect of splenectomy and mono- or combination therapy with rapamycin, the morpholinoethyl ester of mycophenolic acid and deoxyspergualin on cardiac xenograft survival. Transplant Proc 1991, 23:699
88. Zheng B, Shorthouse R, Masek MA, et al: Effects of the new and highly active immunosuppressant, rapamycin, on lymphoid tissues and cells in vivo. Transplant Proc 1991:23:851
89. Eng CP, Gullo-Brown J, Chang JY, et al: Inhibition of skin graft rejection in mice by rapamycin: A novel immunosuppressive macrolide. Transplant Proc 1991, 23:868
90. Keenan RJ, Aeba R, Duncan A, et al: Efficacy of rapamycin and FK506 compared to cyclosporine in preventing acute pulmonary rejection. J Heart Lung Transplant 1991, 10:167
91. Stepkowski SM, Chen H, Daloze P, et al: Rapamycin, a potent immunosuppressive drug for vascularized heart, kidney, and small bowel transplantation in the rat. Transplantation 1991, 51:22
92. Stepkowski SM, Kahan BD: Synergistic effect of cyclosporine and rapamycin on heart and kidney allograft survival. FASEB J 1991, 5:1711 (abstr)
93. Stepkowski SM, Chen H, Daloze P, et al: Prolongation by rapamycin of heart, kidney, pancreas, and small bowel allograft survival in rats. Transplant Proc 1991, 23:507
94. Chen HF, Wu JP, Luo HY, et al: The immunosuppressive effect of rapamycin on pancreaticoduodenal transplantations in the rat. Transplant Proc 1991, 23:2239
95. Waren RP, Lofgren JS, Steinmuller D: Differential survival of heart and skin allografts in inbred rats. Transplant Proc 1973, 5:717
96. Honbo T, Kobayashi M, Hane K, et al: The oral dosage form of FK-506. Transplant Proc 1987, 19:17
97. Todo S, Ueda Y, Demetris JA, et al: Immunosuppression of canine, monkey, and baboon allografts by FK 506. Surgery 1988, 104:239
98. Venkataramanan R, Warty VS Zemaitis MA, et al: Biopharmaceutical aspects of FK-506. Transplant Proc 1987, 19:30 (suppl 6)
99. Chen HF, Wu JP, Luo HY, et al: Reversal of ongoing rejection of allografts by rapamycin. Transplant Proc 1991, 23:2241
100. Auchincloss H: Xenografting: A review. Transplant Rev 1990, 4:14
101. Berenbaum MC: What is synergy? Pharmacol Rev 1989, 41:93
102. Venkataramanan R, Warty VS Zematis MA, et al: Transplant Proc 1988, 20:30
103. Lake KD: Update and management of cyclosporine drug interactions. Pharmacotherapy 1991, 11:1105 (suppl)
104. Venkataramanan R, Jain A, Warty VW, et al: Pharmacokinetics of FK 506 following oral administration: A comparison of FK 506 and cyclosporine. Transplant Proc 1991, 23:931
105. Lorber MI, Wilson JH Harding MW, et al: A comparison of in vivo response to cyclosporine and rapamycin following allogeneic immune challenge. Transplantation 1991, 52:167
106. Beschorner WE, Namnoum JD, Hess AD, et al: Cyclosporin A and the thymus. Am J Pathol 1987, 126:487
107. Beschorner WE, Di Gennaro KA, Hess AD, et al: Cyclosporine and the thymus: Influence of irradiation and age on thymic immunopathology and recovery. Cell Immunol 1987, 110:350
108. Beschorner WE, Olson JL, Hess AD, et al: Cyclosporine-induced cell-mediated injury of the thymic medullary epithelium. Transplantation 1988, 45:797

109. Shinozawa T, Beschorner WE, Hess AD: The thymus and prolonged administration of cyclosporine. Transplantation 1990, 50:106
110. Nalesnik MA, Todo S, Murase N, et al: Toxicology of FK-506 in the Lewis rat. Transplant Proc 1987, 19:89 (suppl 6)
111. Pugh-Humphreys RGP, Ross CSK, Thomson AW: The influence of FK-506 on the thymus: An immunophenotypic and structural analysis. Immunology 1990, 70:398
112. Thomson AW, Pugh-Humphreys RGP, Woo J: The influence of FK 506 on the thymus: Implications of drug-induced injury to thymic epithelial cells. Transplant Proc 1991, 23:947
113. Thomson AW, Pugh-Humphreys RGP: The antilymphocytic properties of FK506 and its influence on the thymic microenvironment, in Ritter M, Kendall M, (Eds): Thymus Update. Harwood, London, England, 1991, pp 93-117
114. Beschorner WE, Hess AD, Shinn CA, et al: Transfer of cyclosporine-associated syngeneic graft-versus-host disease by thymocytes. Transplantation 1988, 45:209
115. Staruch MJ, Dumont FJ, Sigal NH: FK-506 inhibits activation-driven T cell death. FASEB J 1990, 4:2038 (abstr)
116. Bierer BE, Mattila PS, Standaert RF, et al: Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either fk506 or rapamycin. Proc Nat Acad Sci USA 1990, 87:9231
117. Bierer BE, Schreiber SL, Burakoff SJ: The effect of the immunosuppressant FK-506 on alternate pathways of T cell activation. Eur J Immunol 1991, 21:439
118. Turka LA, Linsley PS, Paine III R, et al: Signal transduction via CD4, CD8, and CD28 in mature and immature thymocytes. J Immunol 1991, 146:1428
119. Lin CS, Siekierka JJ, Sigal NH: Differential effects of rapamycin and FK-506 on human T cell activation. FASEB J 1990, 4:2033 (abstr)
120. June CH: Signal transduction in T cells. Curr Opin Immunol 1991, 3:287
121. Crabtree GR: Contingent genetic regulatory events in T lymphocyte activation. Science 1989, 243:355
122. Berry N, Nishizuka Y: Protein kinase C and T cell activation. Eur J Biochemistry 1990, 189:205
123. Burgess KE, Odysseos AD, Zalvan C, et al: Biochemical identification of a direct physical interaction between CD4:p54^{lck} and T_1(TcR)/CD3 complexes. Eur J Immunol 1991, 21:1663
124. Van Lier RAW, Brouwer M, De Groot E, et al: T cell receptor/CD3 and CD28 use distinct intracellular signaling pathways. Eur J Immunol 1991, 21:1775
125. June CH, Ledbetter JA, Lindsley PS, et al: Role of the CD28 receptor in T-cell activation. Immunol Today 1990, 11:211
126. Ledbetter J, Imboden J, Schieven G, et al: CD28 ligation in T-cell activation: Evidence for two signal transduction pathways. Blood 1990, 75:1531
127. Sephajpal PK, Li B, Zanker B, et al: The molecular basis for the synergism between the CD3/$\partial\beta$ T cell receptor and the CD2 antigen-derived signals in promoting T cell proliferation. Transplantation 1991 51:468
128. Van Seventer GA, Shimizu Y, Horgan KJ, et al: Remote T cell co-stimulation via LFA-1/ICAM-1 and CD2/LFA-3: Demonstration with immobilized ligand/mAb and implication in monocyte-mediated costimulation. Eur J Immunol 1991, 21:1711
129. Smith KA: Interleukin-2: Inception, impact, and implications. Science 1988, 240:1169
130. Hatakeyama M, Kono T, Kobayashi N, et al: Interaction of the IL-2 receptor with the *src*-family kinase p56^{lck}: Identification of novel intermolecular association. Science 1991, 252: 1523
131. Dumont FJ, Melino MR, Staruch MJ, et al: The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T cells. J Immunol 1990, 144:1418
132. Dumont FJ, Altmeyer A: Differential modulation of IL-1–induced IFN-gamma production by the immunosuppressive macrolides rapamycin (RAP) and FK-506. Lymphokine Res 1990, 9:574
133. Bansbach CC, Casilio-Lonardo E, Adams LM, et al: Inhibition of human lymphocyte proliferation by rapamycin, a novel immunosuppressant. FASEB J 1991, 4:1768 (abstr)
134. Zeevi A, Eiras G, Duquesnoy R: Immunosuppressive effects of rapamycin and FK506 on human T cells. J Heart Lung Transplant 1991, 10:175
135. Kahan BD, Gibbons S, Tejpal N, et al: Synergistic interactions of cyclosporine and rapamycin to inhibit immune performances of normal human peripheral blood lymphocytes *in vitro*. Transplantation 1991, 51:232
136. Kimball PM, Kerman RH, Kahan BD: Production of synergistic but nonidentical mechanisms of immunosuppression by rapamycin and cyclosporine. Transplantation 1991, 51:486
137. Metcalfe S, Milner J: Phosphatases PP1 and PP2A act after the G0/G1 interface in lymphocyte activation. Immunol Lett 1990, 26:177
138. Metcalfe S, Milner J: Evidence that FK506 and rapamycin block T cell activation at different sites relative to early reversible phosphorylation involving the protein phosphatases PP1 and PP2A. Transplantation 1991, 51:1318
139. Luo H, Chen P, Daloze J, et al: Rapamycin suppresses in vitro immunoglobulin production by human lymphocytes. Transplant Proc 1991, 23:2236
140. Reth M, Hombach J, Wienands J, et al: The B-cell antigen receptor complex. Immunol Today 1991, 12:196
141. Alés-Martínez JE, Cuende E, Martínez-AC, et al: Signalling in B cells. Immunol Today 1991, 12:201
142. Justement LB, Campbell KS, Chien NC, et al: Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science 1991, 252:1839
143. Wicker LS, Boltz RC, Matt V, et al: Suppression of B cell activation by cyclosporin A, FK506 and rapamycin. Eur J Immunol 1990, 20:2277
144. Bierer BE, Schreiber SL, Burakoff SJ: Mechanisms of immunosuppression by FK506. Preservation of T cell transmembrane signal transduction. Transplantation 1990, 49:1168
145. Heitman J, Movva NR, Hiestand PC, et al: FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in *Saccharomyces cerevisiae*. Proc Nat Acad Sci USA 1991, 88:1948
146. Koltin Y, Faucette L, Bergsma DJ, et al: Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl *cis-trans* isomerase related to human FK506 binding protein. Mol Cell Biol 1991, 11:1718
147. Heitman J, Movva NR, Hall MN: Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 1991, 253:905
148. Siekierka JJ, Staruch MJ, Hung SH, et al: FK-506, a potent

novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. J Immunol 1989, 143:1580
149. Siekierka JJ, Hung SH, Poe M, et al: A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 1989, 341:755
150. Siekierka JJ, Wiederrecht G, Greulich H, et al: The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptidyl-prolyl cis-trans isomerase. J Biol Chem 1990, 265:21011
151. Michnick SW, Rosen MK, Wandless TJ, et al: Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. Science 1991, 252:836
152. Moore JM, Peattie DA, Fitzgibbon MJ, et al: Solution structure of the major binding protein for the immunosuppressant FK506. Nature 1991, 351:248
153. Fretz H, Albers MW, Galat A, et al: Rapamycin and FK506 binding proteins (immunophilins). J Am Chem Soc 1991, 113:1409
154. Jin Y-J, Albers MW, Lane WS, et al: Molecular cloning of a membrane-associated human FK506- and rapamycin-binding protein, FKBP-13. Proc Natl Acad Sci USA 1991, 88:6677
155. Touchette N: Immune suppressants signal surprises in T cell activation. J NIH Res 1991, 3:70
156. DeFranco AL: Immunosuppressants at work. Nature 1991, 352:754
157. Liu J, Farmer JD, Lane WS, et al: Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991, 66:807
158. Friedman J, Weissman I: Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: One in the presence and one in the absence of CsA. Cell 1991, 66:799
159. Marks WH, Harding MW, Handschumacher R, et al: The immunochemical distribution of cyclophilin in normal mammalian tissues. Transplantation 1991, 52(2):340
160. Koletsky AJ, Harding MW, Handschumacher RE: Cyclophilin: Distribution and variant properties in normal and neoplastic tissues. J Immunol 1986, 137:1054
161. Freedman RB: Immunosuppression. Convergence of drug action. Nature 1989, 341:692
162. Rosen MK, Standaert RF, Galat A, et al: Inhibition of FKBP rotamase activity by immunosuppressant FK506: Twisted amide surrogate. Science 1990, 248:863
163. Albers MW, Walsh CT, Schreiber SL: Substrate specificity for the human rotamase FKBP: A view of FK506 and rapamycin as leucine-(twisted amide-)proline mimics. J Organ Chem 1990, 55:4984
164. Bierer BE, Somers PK, Wandless TJ, et al: Probing immunosuppressant action with a nonnatural immunophilin ligand. Science 1990, 250:556
165. Sigal NH, Dumont F, Durette P, et al: Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? J Exp Med 1991, 173: p. 619-628
166. Flanagan WM, Corthésy B, Bram RJ, et al: Nuclear association of a T-cell transcription factor blocked by FJ-506 and cyclosporin A. Nature 1991, 352:803
167. Standaert RF, Galat A, Verdine GL, et al: Molecular cloning and over expression of the human FK506-binding protein FKBP. Nature 1990, 346:671
168. van Duyne GD, Standaert RF, Karplus PA, et al: Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. Science 1991, 252:839
169. Pardee AB: G1 events and regulation of cell proliferation. Science 1989, 246:603
170. Stern JB, Smith KA: Interleukin-2 induction of T-cell G_1 progression and *c-myb* expression. Science, 1986, 233:203
171. Cyert MS, Thorner J: Putting it on and taking it off: Phosphoprotein phosphatase involvement in cell cycle regulation. Cell 1989, 57:891
172. Alexander DR, Cantrell DA: Kinases and phosphatases in T-cell activation. Immunol Today 1989, 10:200
173. Ashwell JD: Pas de deux. Curr Biol 1991, 1:111
174. Ledbetter JA, Schieven GL, Uckun FM, et al: CD45 cross-linkage regulates phospholipase C activation and tyrosine phosphorylation of specific substrates in CD3/Ti stimulated T cells. J Immunol 1991, 146:1577
175. Marx J: The cell cycle: Spinning farther afield. Science 1991, 252:1490
176. Balter M: Cell cycle research: Down to the nitty gritty. Science 1991, 252:1253
177. Woodgett JR: Powering the cell cycle. Curr Biol 1991, 1:106
178. Norbury C, Nurse P: Cyclins and cell cycle control. Curr Biol 1991, 1:23
179. Furukawa Y, Piwnica-Worms H, Ernst TJ, et al: *cdc2* gene expression at the G1 to S transition in human T lymphocytes. Science 1990, 250:805
180. Furukawa Y, DeCaprio JA, Freedman A, et al: Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells. Proc Nat Acad Sci 1990, 87:2770
181. Chisholm PM, Bevan DJ: T cell activation in the presence of cyclosporine in three in vivo allograft models. Transplantation 1988, 46:80S (suppl)
182. Cox DR: Analysis of Binary Data. London, England, Chapman and Hall, 1970
183. McCullagh P: Generalized Linear Models (ed 2). London, England, Chapman and Hall, 1989