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NEW IMMUNOSUPPRESSIVE DRUGS:

MECHANISMS OF ACTION AND EARLY CLINICAL EXPERIENCE

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INTRODUCTION

New immunosuppressive drugs with distinct and diverse modes of action are currently subjects of intense interest both in basic cell science and in the field of organ transplantation. They excite immunologists and molecular biologists interested in using these agents as probes to study the regulation of lymphocyte activation and growth. They also represent important developments both in the pharmaceutical industry and to clinicians interested in their prospective therapeutic applications. When viewed over a 40-year perspective (1950-1990), the introduction of a new immunosuppressive agent into widespread clinical use for the prevention or control of organ graft rejection has been an infrequent event. Significantly, the immunosuppressive drugs used traditionally to suppress allograft rejection have been by-products of the development of anti-cancer (anti-proliferative) agents (eg. azathioprine) or anti-inflammatory agents (such as corticosteroids). The fortuitous discovery of the fungal product cyclosporin A (CsA), - an immunosuppressant with a selective inhibitory action on T lymphocyte activation and proliferation, was a result of the screening of natural products for antibiotic activities. Only very recently, with the advent of FK 506 (a drug with a similar action to CsA) has an immunosuppressant been introduced which embodies the results of a deliberate search for

new, selective anti-lymphocytic agents with acceptable efficacy and toxicity profiles.

The introduction in 1983 of CsA into widespread clinical practice for the prophylaxis of organ allograft rejection was followed by worldwide improvement in the results of organ transplantation. This set the scene for the introduction of newer, candidate immunosuppressive drugs offering a combination of specificity of action and comparative safety. The potential, additional applications of new drugs in the treatment of various non-transplant disorders, as evidenced for example, by the efficacy of CsA in psoriasis and certain other autoimmune diseases, has provided additional incentives within the pharmaceutical industry for the development of new immunosuppressive agents.

Although our knowledge of key molecular events underlying immune cell activation has advanced enormously in recent years, we still stand well short of a complete understanding of the sequence of events between antigen recognition, lymphocyte activation and cell proliferation. The most recent developments concerning the actions of new immunosuppressive drugs have however, shed light on several biochemical events crucial for immune function. Further understanding of these fundamental processes may assist in future drug design. The new immunosuppressive drugs featured in this chapter represent a range of molecules which act at distinct sites in lymphocyte activation and proliferation (Table 1). Thus FK 506

inhibits cytokine gene expression, Rapamycin (Rapa) blocks the actions of cytokines but not cytokine production, mycophenolic acid (the active moiety of RS61443) and brequinar inhibit DNA synthesis, whilst deoxyspergualin (DSG) interferes with cell maturation. The purpose of this chapter is to review the modes of action and immunosuppressive properties of these new agents and to provide an up-to-date account of early clinical experience with each agent, where that is possible.

INHIBITION OF CYTOKINE SYNTHESIS

The hallmark of CsA and the principal factor accounting for its prominence as first choice conventional agent for the control of graft rejection is its capacity to inhibit selectively, cytokine gene expression in $CD4^+$ T helper (T_H) lymphocytes. This property is shared by the new drug FK 506.

FK 506

The macrolide antibiotic FK 506 ($C_{44}H_{69}NO_{12}H_2O$;mw822D) is a fermentation product of the fungus Streptomyces tsukubaensis. It was identified in 1982-83 by the Fujisawa Pharmaceutical Co. Ltd., in Osaka, Japan during routine screening of natural products for specific inhibitory effects on mixed lymphocyte reactions (MLR). Although FK 506 is totally distinct in structure (Fig. 1) from CsA, the two drugs have a remarkably similar molecular action; FK 506

is, however, ten- to one hundred- fold more potent. The recently published Proceedings of the First International Congress on FK 506[1] provide a comprehensive review of the molecular action, pharmacokinetics, immunosuppressive activities and toxicity of this powerful new immunosuppressant.

THE MOLECULAR ACTION OF FK 506

Like CsA, FK 506 acts primarily but not exclusively on T cells. It blocks $CD4^+$ T helper (T_H) lymphocyte activation and cytokine production with consequent inhibitory effects on other T and non-T cell components of the immune system. It inhibits T lymphocyte activation mediated by the T-cell receptor (TCR)-CD3 complex and also via the cell surface molecule, CD2.[2] Induction of T-cell proliferation, IL-2 production and apoptosis (programmed cell death) are all sensitive to both FK 506 and CsA. When administered within one hour (and up to 6 hours) of the activation stimulus, FK 506 is very effective in suppressing alloantigen-induced lymphocyte proliferation <u>in vitro</u>, at concentrations 100-fold lower than effective concentrations of CsA (Table 2). FK 506 also inhibits the generation of cytotoxic and suppressor T cells in human MLR, but does not affect antigen recognition by cytotoxic T cells, or the mechanism by which target cells are destroyed.

There is little evidence that of FK 506 directly affects the functions of accessory cells. Recently, however, Keicho etal[3] found that FK 506 partially inhibited IL-1α release from phorbol myristate acetate (PMA)-stimulated macrophage-like U937 cells and from lipopolysaccharide (LPS) activated human monocytes. Earlier, Woo etal[4] showed that concentrations of FK 506 which strongly inhibited antigen (purified protein derivative; PPD)-induced human T cell proliferation had little effect on antigen processing or presentation by human blood monocytes. Functions of other leukocytes, however, are affected by FK 506, although this may depend on the nature of the stimulus. Thus, FK 506 (like CsA) selectively inhibits calcium-dependent signalling pathways in murine B cells activated using anti-IgM or low dose anti-IgM plus IL-4.[5,6] Interestingly, both FK 506 and CsA inhibit proinflammatory mediator release from human basophils and rat mast cells,[7,8] as well as transcription of several cytokine genes, including IL-3 and IL-5. These actions may contribute to some of the drugs' therapeutic effects in graft rejection.

In T-cells, FK 506 disrupts an unknown step in the transmission of signals from the cell membrane TCR to genes that coordinate the immune response. The drug acts at a step distal to cell membrane receptors and second messengers but proximal to transcriptional activation of early genes. Experiments designed to ascertain the influence of FK 506 on very early events prior to

gene transcription following binding of antigen to the TCR, have shown that the drug does not affect Ca^{++} mobilization, phosphatidylinositol turnover, activation of tyrosine kinases or phosphatase or the activation of serine/threonine kinases. Both FK 506 and CsA, however, strongly and specifically inhibit expression of early T cell activation genes[9] encoding IL-2 - the main growth factor for T cells, IL-3, IL-4, IFN-γ, GM-CSF and c-myc. Recently, two subsets of murine $CD4^+$ T cells (T_{H1} and T_{H2}) have been identified on the basis of their cytokine secretion and functional profiles[10] and there is growing evidence that such functional subsets also exist in man. T_{H1} cells produce IL-2, IFN-γ, and TNFß, whereas T_{H2} cells secrete IL-4, IL-5, and IL-10, but not IL-2 or IFN-γ. There is evidence that, in vitro, FK 506 may spare IL-10 (cytokine synthesis inhibitory factor) gene transcription by cloned murine T_{H2} cells, whilst suppressing concomitant IL-4 mRNA production.[11] Thus, differential interference with T cell cytokine gene expression and crossregulation of T_{H1} cell (IL-2 and IFN-γ production) may be important mechanisms whereby FK 506 inhibits immune cell activation and maintains immunosuppression.

Insight into the molecular action of FK 506 and CsA has come from studies of their specific, intracellular cytosolic receptors or "immunophilins," - FK 506 binding protein (FKBP) and cyclophilin, respectively.[12] FKBP-12 and cyclophilin A are the predominant members of the FKBP and cyclophilin families of

"immunophilins." Both FKBP and cyclophilin are peptidyl-prolyl cis-trans isomerases (PPIases) which catalyze the slow cis-trans isomerization of ala-pro bonds in oligopeptides and accelerate slow, rate-limiting steps in the folding of several proteins.[13] Although binding of the drug by its respective immunophilin inhibits isomerase activity, recent results indicate that inhibition of isomerase activity is not the relevant effect of the immunosuppressants. It now appears that FK 506 and CsA function as prodrugs and that their immunosuppressive effects result from the formation of active complexes between the drug and its respective isomerase. These complexes interfere with signal transduction within the cell. Recently, Schreiber and his colleagues have shown that the complexes of FK 506 and FKBP and of CsA and cyclophilin bind specifically to three polypeptides, - calmodulin, and the two subunits of calcineurin (a Ca^{++}-activated, serine-threonine protein phosphatase).[14] In each case, FK 506 or CsA promotes the interaction of the normally non-interacting immunophilin and calcineurin (Fig. 2). The drug-immunophilin complexes block the Ca^{++}-activated phosphatase activity of calcineurin, which appears to be the target of these complexes.[14] In contrast, neither FK 506, CsA, FKBP, nor cyclophilin alone inhibits the phosphatase activity of authentic calcineurin.[14]

A second key observation reported by Crabtree and colleagues is that the drug-immunophilin complexes block Ca^{++}-dependent

assembly of a functional gene transcription activator (NF-AT) by inhibiting translocation of the pre-existing component of NF-AT from the cytoplasm to the nucleus.[15] The nuclear component of NF-AT is transcriptionally inactive in all cells other than activated T lymphocytes and is induced by signals from the TCR. Its appearance is not blocked by FK 506 or CsA. Current thinking is that FK 506 and CsA block dephosphorylation by calcineurin of the cytoplasmic component of NF-AT which is required for its translocation to the nucleus. In the absence of both nuclear and cytoplasmic components, binding of NF-AT to DNA and transcriptional activation of the IL-2 gene and other genes are suppressed (Fig. 3). Whilst transcription directed by NF-AT is blocked in T cells treated with FK 506 or CsA, little or no effect is observed on other transcription factors, such as NF-KB or AP-1.[15]

EFFECTS OF FK 506 ON IMMUNE RESPONSES

Kino and colleagues first showed that treatment of mice with FK 506 suppressed the production of antibody plaque-forming (B) cells against sheep red blood cells (SRBC), the generation of delayed-type hypersensitivity (DTH) responses against methylated bovine serum albumin and the development of graft-vs-host reactions.[16] Short courses of FK 506 also inhibit DTH responses to SRBC in mice[16] and suppress the production of serum anti-SRBC antibody and anti-MHC class I alloantibody in rats.[17]

The immunosuppressive action of FK 506 however, is not fully understood and has not been studied extensively. Its presumed mechanism of action in vivo is deduced from results of in vitro experiments. A possible role of suppressor cells in FK 506-treated animals has been implied in a limited number of studies. Thus, flow cytometric analyses of lymphocytes of rats treated with a short course of FK 506 beginning on the day of immunization revealed a transient increase in splenic $CD8^+$ cells, with a concomitant decrease in $CD4^+$:$CD8^+$ ratios.[18] Interestingly, a role for suppressor cells in FK 506-treated animals was further implicated by the transfer of unresponsiveness using spleen cells from FK 506 treated, long-term allograft survivors to naive recipients,[19] and by the dependence on the spleen for the immunosuppressive properties of FK 506 in mice.[20] On the other hand, Yoshimura etal,[21] demonstrated that FK 506 prevented the development of suppressor cells in vitro. Thus the role of suppressor cells in inducing immunosuppression in FK 506-treated animals is unresolved. There is, however, little doubt that immunosuppression in vivo is a direct result of inhibition of $CD4^+$ helper T cells. FK 506 inhibits T cell maturation in mice, as evidenced by reductions in both $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes in normal animals[22] and by decreases in mature $CD4^+$ cells in the thymus and peripheral lymphoid tissue following syngeneic bone marrow transplantation.[23] Flow cytometric analyses have revealed that, in

rats, the increase in number of circulating, activated ($IL\text{-}2R^+$) $CD4^+$ lymphocytes during a SRBC-induced immune response is reduced significantly.[24]

FK 506 AND EXPERIMENTAL ORGAN TRANSPLANTATION

RODENTS

In rats, long-term heterotopic cardiac allograft survival can be achieved by treatment with FK 506 at 0.32mg/kg, i.m. or 1.0mg/kg, p.o. in the F344 to WKA strain combination,[19,25] or 1.28mg/kg i.m. in the ACI ($RT1^a$) to Lewis ($RT1^l$) combination.[26] Using a different strain combination, DA ($RT1^a$) to PVG($RT1^u$), however, Lim etal[27] found that long-term graft survival was not observed and median survival time (MST) fell between 22 to 30 days postoperatively.

Using the ACI to Lewis combination, it was further demonstrated that a temporary, donor-specific unresponsiveness was developed in these long-term heart graft survival recipients; a graft from a third party donor was readily rejected, while a second, homologous graft[26,28] had prolonged survival without any further administration of FK 506.[26,28] The effective administration of FK 506 in preventing cardiac allograft rejection seems to be schedule-dependent as there is a shorter MST following reduction of treatment frequency per week.[20,26,28] In addition, maximum prolongation of cardiac allograft survival was reported when

treatment was given from days 4 to 6 postoperatively in the ACI to Lewis strain combination,[26] at which time signs of cardiac graft rejection begin to occur.

FK 506 is also effective in prolonging rodent skin, liver islet, small intestine and limb allografts and in the prevention or reversal of graft-versus-host disease following bone marrow transplantation.

DOGS

As with studies in the rat, FK 506 was found to be effective in controlling allograft rejection in dogs.[29,30] Its side effects in dogs, however, including anorexia and vasculitis caused considerable concern.[31,32] The vascular lesions were shown to be relatively species-specific and not strongly associated, if at all, with FK 506.[33,34] At 1.0 mg/kg, p.o., and 0.16 mg/kg, i.m., FK 506 induced long-term renal graft survival with healthy functioning grafts.[31] Other groups however, have reported a less significant improvement.[29,30,33,34,35,36] Combinations of suboptimal, ineffective doses of FK 506 and CsA resulted in significant prolongation of renal allograft survival,[29,34,37] with reduction in the incidence of anorexia associated with FK 506 and the toxicity attributed to CsA.[29,34,37] Withdrawal of FK 506 usually resulted in the reoccurrence of rejection.[34] FK 506 rescue treatment could reverse 80% of ongoing rejection in renal allografts.[34,36]

Apart from the prolonging renal allograft survival, a short course of FK 506 treatment can also prolong liver[34,36,38] pancreas[39] and pancreaticoduodenal[40] graft survival in dogs at dosages similar to those that prolong renal allograft survival.

NON-HUMAN PRIMATES

Renal transplants performed on cynomolgus monkeys[34] and baboons[34,35,41,42] demonstrated that FK 506 was effective in graft prolongation. Compared to the doses used to inhibit rejection in rats and dogs, a higher dose of FK 506, (12-18 mg/kg/d, p.o.), is necessary to achieve renal graft prolongation in baboons.[41] This may be due to the difference in sensitivity to FK 506 of lymphocytes from different species, since MLR reactivity of baboon lymphocytes appears to be more resistant to the effect of FK 506.[42,43] Induction of tolerance is not seen with respect to renal grafts, as termination of FK 506 treatment leads to reoccurrence of rejection.[36,41] However, a 3-day course of treatment with FK 506 (1 to 2 mg/kg) can effectively overcome the rejection.[36,41,44]

INHIBITION OF CYTOKINE ACTION

RAPAMYCIN (RAPA)

The macrolide antibiotic Rapa ($C_{51}H_{79}NO_3$; mn 914.2D) (Fig 1) is a fermentation product of the soil fungus Streptomyces hygroscopicus isolated by Ayerst Research Laboratories, Montreal in

1975. Its characteristics, including the capacity to inhibit growth of Candida albicans, were first described by Seghal etal in 1975.[45,46] Interest in the powerful T-cell inhibitory properties of Rapa was rekindled in the late 1980s, following accounts of the immunosuppressive efficacy and safety of the structurally-related macrolide FK 506 in experimental and clinical organ transplantation. At the same time, Rapa, like FK 506, was identified as an important new investigational tool for the analysis of molecular events underlying signal transduction in T lymphocytes. The mode of action of Rapa, its immunosuppressive properties and its effects on organ allograft survival have been topics of several recent reviews.[47-50]

THE MOLECULAR ACTION OF RAPA

Rapa is a more powerful inhibitor of T lymphocyte proliferation than CsA. It inhibits murine and human T and B cell activation by a variety of pathways, including those insensitive to FK 506 or CsA, e.g. IL-2-mediated proliferation of IL-2 dependent T-cell lines, activation of human T-cells by phorbol ester (PMA) and anti-CD28 (Table 3).[51,52] Rapa also inhibits activation of murine or human B cells by lipopolysaccharide (LPS)[51] or pokeweed mitogen,[53] respectively. It binds to the same cytosolic receptor (FKBP) as FK 506, but not to cyclophilin.[12] In contrast to FK 506 and CsA, Rapa affects both TCR/CD3 and protein kinase C (PKC) activation pathways

within T-cells.[51,52] Moreover, delay in the addition of Rapa to cultures of T cells for up to 24 hr does not prevent its inhibitory effect on cell proliferation. This contrasts with the effects of FK 506 and CsA, which exhibit no suppressive activity when added more than 3-6 hours after the start of cultures, suggesting that Rapa principally affects later events in T cell activation. It is also consistent with work by Tocci etal.[9] who showed that, in contrast to FK 506, which blocked mRNA expression for the early activation genes IL-2, IL-3, IL-4, IFN-γ, TNFα, GM-CSF and c-myc, Rapa increased expression of these genes by approximately two-fold. Also, in contrast to FK 506, IL-2R expression was not inhibited by Rapa. Scatchard analysis of the interaction between radiolabeled, recombinant IL-2 (r^{125}I-IL-2) and interleukin-2 receptor (IL-2R) shows that Rapa does not affect either the avidity or number of IL-2R binding sites.[54] Although Rapa does not interfere with radioligand binding, it may retard the intracellular incorporation of r^{125}I-IL-2/IL-2R complexes.[54] The sites of action of FK 506 and Rapa are depicted in Fig. 4. Rapa appears to inhibit cell cycle progression at some point in G1.[53,55] Cytokine-induced proliferation of lymphoid cell lines has been used as a model for distinguishing the modes of action of FK 506 and Rapa. Rapa inhibits not only IL-2- but also IL-4-induced proliferation of CTLL and D10.G4 cell lines.[52] Growth of an IL-6-dependent line (MH60.BSF-2) is also inhibited by Rapa.[56] The mechanisms whereby Rapa causes these

inhibitory effects are uncertain and remain to be elucidated. Despite its structural similarity to FK 506 and its binding to and inhibition of FKBP, Rapa interferes with a distinct set of Ca^{++}-independent signalling pathways, including the pathway emanating from the IL-2R in activated T cells. Complexes of FKBP-Rapa fail to bind and inhibit calcineurin phosphatase activity[14] (Fig. 2) and the target of these complexes remains to be defined. Rapa does not block translocation of the cytoplasmic component of NF-AT to the nucleus after T cell activation. This is consistent with its failure to block NF-AT binding activity and NF-AT directed transcription.[57]

RECIPROCAL ANTAGONISM BETWEEN RAPA AND FK 506 IN VITRO

FK 506, but not Rapa, inhibits T cell activation initiated via the TCR. Rapa on the other hand, inhibits IL-2-induced T cell proliferation, whereas FK 506 does not. These two distinct signal transmission pathways are blocked by complexes formed between FKBP and either FK 506 or Rapa. The dissociation constant of Rapa to FKBP and of FK 506 to FKBP are quite similar. An excess of Rapa, however, is required to reverse FK 506-induced inhibition of transcriptional activation of NF-AT, IL-2 mRNA induction, IL-2 production or apoptosis.[52,58] Similarly, an excess of FK 506 is needed to revert Rapa-mediated inhibition of IL-2-induced T cell proliferation. Neither FKBP binding nor inhibition of the rotamase

activity of FKBP alone (both FK 506 and Rapa inhibit rotamase activity) is sufficient to explain the action of these drugs. These observations demonstrate that FK 506 and Rapa antagonize each other's biological activity and physically interact with a common receptor site. CsA on the other hand, acts at an intracellular site distinct from the target of FK 506 or Rapa.

SYNERGY BETWEEN RAPA AND CsA AND ANTAGONISM BETWEEN FK 506 AND CsA IN VITRO

Kahan and his colleagues have reported mutually synergistic interactions between Rapa and CsA both *in vitro*[53,56] and *in vivo* (see below). Rapa augments the inhibitory effects of CsA on human PBL activation by PHA, anti-CD3 monoclonal antibody, and MLR. Rapa also enhances the capacity of CsA to suppress cytotoxic cell generation and precursor frequency during alloactivation *in vitro*. Moreover, CsA potentiates the inhibitory effects of Rapa on proliferation of IL-2 and IL-6-dependent cell lines.[56]

In contrast, the same group has observed antagonism between low doses of FK 506 and CsA, using the same experimental in vitro systems and in the in vivo Buffalo→Wistar-Furth cardiac allograft model.[59]

RAPA AND EXPERIMENTAL ORGAN TRANSPLANTATION

Rapa has been tested in a variety of experimental transplantation models and has been shown to suppress acute rejection of kidney, heart, skin and small bowel allografts in rodents, pigs, dogs or primates (reviewed).[48] Rapa in a more potent inhibitor than CsA of non-vascularized foetal heart allograft survival in mice (BALB/c→C3H;H-2^d→H-2^k). A dose of 3mg/kg/day i.p. for 14 days prolongs graft survival from 10.6 to 146 days. Depending on the drug formulation, Rapa is also a potent inhibitor of vascularized heterotopic allograft survival in rats. Thus, Stepkowski et al.[60] showed that given by continuous intravenous infusion via osmotic pumps, Rapa (0.8mg/kg in polyethylene glycol for 14 days) extended cardiac graft survival (Buffalo-Wistar Furth) from 6.5 ± 0.5 to 80 ± 3.6 days. Similarly, a 14-day i.v. Rapa infusion with 0.8mg/kg prolonged the survival of Buffalo kidney allograft recipients from 11.6 ± 1.5 to 90.2 ± 62.4 days. A continuous course of 2 mg/kg Rapa protected kidney allografts in pigs[61] but the same regimen was severely toxic in dogs.

A strong synergistic effect of low doses of Rapa and CsA has been reported by Kahan et al,[56] using the Buffalo→Wistar Furth rat cardiac allograft model. Thus, minimally effective doses of Rapa (0.02 mg/kg i.v.) and CsA (2 mg/kg p.o.) when combined, allowed 100% cardiac graft survival beyond 50 days in all graft recipients. Evidence has also been cited[54] of combined Rapa/CsA/RS-61443

treatment prolonging mouse heart allograft survival from 18 to 140 days. It has also been reported by Morris et al.[62] that combination of Rapa with FK506 acts synergistically to prolong mouse heart allograft survival. The latter data are particularly interesting as they are not consistent with reports that, with respect to T cell activation in vitro, Rapa and FK506 are reciprocal antagonists. This apparent discrepancy may be explained by differing relative concentration levels of the two drugs, since, in vitro, a 100-fold molar excess of one drug is required for exhibition of antagonism.

INHIBITION OF DNA SYNTHESIS

MIZORIBINE (MZB) = BREDININ

Mizoribine (MZB; mw=259Da) (Fig. 5) is an imidazole nucleoside antibiotic, isolated from the soil fungus Eupenicillium brefeldianum. Like mycophenolic acid (MPA), MZB inhibits the activity of inosine monophosphate dehydrogenase (IMPDH) in mammalian cells and its growth inhibitory effects are reversed by guanine and guanine monophosphate. Amongst its reported effects on the immune system, which are in accordance with its molecular action, are failure to inhibit early events in T cell activation, including mRNA levels for c-myc, IL-2, c-myb, histone and cdc2 kinase.[63] Cytokine (IL-2) production and cell surface IL-2R expression induced by alloantigens, anti-CD3 monoclonal antibodies

or pharmacologic mitogens are also unaffected. MZB prevents lymphocyte proliferation in vitro, suppresses humoral immunity to both T-dependent and T-independent antigens and impairs DTH responses in experimental animals. MZB has been reported to exhibit equal potency to azathioprine but little bone marrow suppression or hepatotoxicity. It is therefore, like MPA, a candidate drug to replace azathioprine in clinical immunosuppressive drug therapy. MZB suppresses allograft rejection in rodents and dogs and exhibits strong, synergistic effects when combined with CsA to prolong graft survival. Because of reduced side effects compared with azathioprine, it has been used for some years in combination with CsA and steroids to suppresses renal transplant rejection in patients in Japan (see below).

RS-61443 AND MYCOPHENOLIC ACID (MPA)

The antipurine RS-61443 is a semi-synthetic derivative (morpholinoethylester) of mycophenolic acid (MPA) (Fig. 5), and may hold considerable promise as an adjunctive agent administered (in place of azathioprine) in combination with current, standard immunosuppressive drugs (CsA and corticosteroids).[64] It is a noncompetitive inhibitor of IMPDH, the rate controlling enzyme in the de novo biosynthesis of guanine nucleotides. Compared with resting lymphocytes, activated T- or B- cells exhibit marked increases in IMPDH activity and in the production of guanine

nucleotides, which are essential for nucleic acid and protein synthesis. Inhibition of DNA synthesis by MPA causes accumulation of cells at the G1-S interface of the cell cycle. MPA suppresses human T and B cell DNA synthesis stimulated by mitogens or alloantigens and inhibits antibody production both in vitro and in vivo. MPA also prevents generation of allospecific, cytotoxic T cells.[65,66]

RS-61443 is rapidly hydrolysed to produce free MPA, which is the active agent. It has a highly selective, antiproliferative effect restricted to T- and B cells. Since RS-61443 inhibits both T and B-cell proliferation and antibody production and since it has been reported to be neither nephro-, hepato, nor myelotoxic, it may represent a more effective and safe alternative to azathioprine for the control of graft rejection. Significantly, RS-61443 also strongly suppresses B-cell memory responses in vitro. Morris and his colleagues[67] have shown that RS-61443 prolongs heterotopic mouse or heart allograft survival, including graft survival in sensitized rats. Short-term treatment induced a state of donor-specific tolerance. RS-61443 is also effective in prolonging heart xenograft (hamster to rat) survival, particularly when combined with a minimally effective dose of deoxyspergualin (DSG) (see below) and recipient splenectomy. In dogs, RS61443 monotherapy (40 mg/kg/day) markedly prolongs renal allograft survival, but is associated with gastrointestinal toxicity. Triple therapy

consisting of RS-61443 (20mg/kg/day), CsA (5mg/kg/day) and methylprednisolone (0.1mg/kg/day) prolonged renal allograft survival from 8 days (untreated controls) to 122 days, without major side effects (including bone marrow suppression) or infectious complications.[68] Moreover, high-dose therapy with RS 61443 reversed acute kidney allograft rejection in dogs.[69]

BREQUINAR SODIUM (BQR)

The antimetabolite brequinar sodium (BQR) (Fig. 5) is a novel, quinoline carboxylic acid analogue, with broad anti-tumour activity in mice. Its anti-cancer properties in man are currently under investigation. The mechanism of action of BQR in tumour cells is believed to involve inhibition of dihydroorotate dehydrogenase, an enzyme in de novo pyrimidine biosynthesis, resulting in depletion of precursors required for RNA and DNA synthesis. Recent studies have revealed that BQR is effective in inhibiting MLR and cell-mediated immunity in mice. It has also been shown to be very effective either alone or in combination with CsA, at subtherapeutic doses, in the prolongation of experimental heart, liver or kidney allograft survival in the rat (ACI→Lew). The potency of BQR in experimental organ transplantation is similar to that of CsA.[70] Treatment of kidney or liver allograft recipients with BQR for 30 days was sufficient to induce indefinite survival of the graft. In long-term liver allograft survivors, the

unresponsiveness was donor-specific. With respect to preclinical toxicity evaluation, both the bone marrow and gut appear to be targets, especially in the dog. Based on these findings, BQR may prove a valuable new adjunctive immunosuppressive agent for treatment of organ graft rejection.

INHIBITION OF CELL MATURATION

DEOXYSPERGUALIN (DSG)

15 - deoxyspergualin (DSG) is a semi-synthetic polyamine (mw 496.9; $C_{17}H_{37}N_7O_3$3HCL) (Fig. 6) with anti-tumour activity. The bacterium Bacillus lactosporus synthesizes a natural product, spergualin, which, when synthetically hydroxylated, is converted to DSG. It exhibits a novel spectrum of immunosuppressive activity in experimental animals and has been shown to be effective in allogeneic and xenogeneic transplantation models in non-human primates. In man, it is effective (either alone or in combination with other agents) as rescue therapy in renal transplantation (see below).

Elucidation of the mode of action of DSG in vitro has been slow, due largely to drug instability at neutral pH and hydrolysis in culture medium. It is evident, however, that DSG may have a predominant effect on monocyte/macrophage function, including inhibition of oxidative metabolism, lyosomal enzyme synthesis, IL-1 production and cell surface expression of MHC class II (Ia)

antigens. Apart from influencing monocytes/macrophages, DSG also inhibits in vivo generation of cytotoxic T cells, either directly or indirectly. DSG does not affect IL-2 production. On the other hand, the recovery of secondary, cytotoxic T cell activity (that is susceptible to DSG) following addition of IFN-γ suggests that suppression of IFN-γ production may be the main effect of DSG on T cell populations.

DSG suppresses LPS-stimulated B cell blastogenesis and in a hamster-to-rat xenograft model, antibody-mediated first-set xenograft rejection and hyperacute rejection. It thus appears that DSG may also affect B-cell activation, differentiation and maturation, leading to inhibition of antibody production.

The precise biochemical action of DSG is uncertain. It is well-known however, that high levels of naturally occurring polyamines decrease levels of ornithine decarboxylase, thus elevating intracellular ornithine concentration which, in turn, is known to suppress cytotoxic T-cell differentiation, but not IL-2 secretion. These properties of DSG and its effects on the immune system have been reviewed recently.[71]

In combination with rabbit antithymocyte globulin (RATG), DSG is superior to treatment with either agent alone in prolonging hamster xenograft survival in the rat. Optimal graft prolongation (21 days versus untreated control of 3 days) was achieved with RATG and DSG (2.5mg/kg/day) by continuous infusion.[72] The combination

of DSG (10mg/kg/day) with splenectomy performed 1 week before transplantation, resulted in survival of mouse heterotopic heart allografts for 17.8 days, compared with 4.7 days and 6.8 days respectively, with splenectomy or DSG alone.[73]

15-DSG has been evaluated in abdominal, heterotopic cardiac transplantation in the cynomolgus monkey. Daily administration of "low-dose" DSG (2mg/kg i.m.) beginning on the morning of operation, did not affect either graft survival time or rejection grade, whereas "high dose" DSG (7.5mg/kg i.m.) caused systemic toxicity.[74]

CLINICAL EXPERIENCE WITH NEW IMMUNOSUPPRESSIVE AGENTS

As might be expected, clinical trials of these new immunosuppressive agents have lagged behind the laboratory studies. There are however, several preliminary reports concerning FK 506, RS-61443, MZB, and DSG, and a number of ongoing or planned studies of these and other agents. This section of the chapter will serve to summarize the current status of these drugs in clinical kidney transplantation.

FK 506

PITTSBURGH EXPERIENCE

FK 506 was first used in clinical kidney transplantation in March, 1989. By the time of the first report, which included the patients transplanted by January, 1990, 36 cases had been done

under primary therapy with FK 506 and steroids.[75] The second report, which included cases transplanted through June, 1990, described 66 cases.[76] By the time of the First International Conference on FK 506, in August, 1991, some 240 cases were available for analysis.[77] At this latter time, a continuous i.v. infusion of 0.1mg/kg FK 506 per day was used until patients were on a solid diet, at which point an oral dose of 0.15mg/kg twice daily was started. The results and conclusions were similar throughout this period and showed a one year actuarial patient and graft survival of 90% and 74%, respectively. These results were comparable to a nearly equivalent number of patients transplanted within roughly the same time frame under CsA-based immunosuppression (Table 4). Both groups were unselected and reflected a relatively high percentage of patients undergoing retransplantation and/or having a high level of pre-formed antibodies. There were a few notable advantages to FK 506 described in all of these initial reports. First, a substantial fraction of successfully transplanted patients, between 40 and 50%, were able to be weaned off steroids. Second, an equally large number of patients were managed without antihypertensive medications. Finally, the serum cholesterol levels were significantly lower in the FK 506 patients than in the patients on CsA. Within this most recent report was a subset of data on

patients who were randomized to receive CsA or FK 506. The results mirrored those of the larger group.

In the subgroup of patients in which transplantation had been historically most successful, ie. living related donor transplantation, and in pediatric transplantation, FK 506 was associated with extremely good results. In the first 28 living related kidney transplantations under FK 506, patient and graft survival were 100%; over 80% of the patients who were more than 5 months post-transplantation were off prednisone.[78] Of the first 20 pediatric kidney transplantations under FK 506, only 1 was lost, to recurrent hemolytic uremic syndrome, and over 1/2 of the children were taken off prednisone.[79,80] Steroid withdrawal may be particularly important for long-term growth and development in children.

On the basis of these studies, which demonstrated that FK 506 was at least comparable to CsA and was possibly better on the basis of secondary issues, a new randomized trial was begun comparing FK-506/prednisone with FK 506/azathioprine/prednisone. Although this study is still in progress, preliminary analysis (March 1992) indicates an early engraftment rate of 95%, with only 2 graft losses in the first 54 cases and no patient deaths.[81] While this study will need to be completed and an analysis performed over a longer time period, these preliminary data suggest that there is a learning curve associated with the use of FK 506 in kidney

transplantation. It may well be that FK 506 will, over time, prove to be superior to CsA.

JAPANESE EXPERIENCE

The other significant clinical experience to date with FK 506 in kidney transplantation has been accumulated in a multicenter early Phase 2 trial in Japan.[82] The first report was on 37 cases, most of which were with living related donors. Although 100% patient and graft survival was obtained, some 30% of the patients were withdrawn from FK 506 because of side effects. At present, a second multicenter late Phase 2 trial is underway in Japan.

Recently, a multicenter randomized trial was started in the United States.

RESCUE

The other use of FK 506 in kidney transplantation has been as rescue therapy in cases of rejection or proteinuria. About 70% of patients with unremitting acute rejection were successfully salvaged.[83] These patients were referred after failing under conventional immunosuppression and both steroid and antilymphocyte therapy for rejection. In the transplant-related nephrotic patients, over 1/2 of the patients without chronic rejection had reduction or elimination of their proteinuria.[84] FK 506 rescue is

generally unsuccessful in situations where the predominant picture is one of chronic rejection.

SIDE EFFECTS

There are three basic categories of side effects of FK 506, -nephrotoxicity, neurotoxicity, and diabetogenicity. Nephrotoxicity is comparable in degree to that seen with CsA, and even the histologic picture is remarkably similar.[85] As with CsA the nephrotoxicity is largely reversible with dosage reduction,[75,77] although there may be an irreversible chronic component seen with long-term toxicity, again similar to the picture seen with CsA. Nephrotoxicity can be distinguished from rejection rather easily by biopsy and can often be inferred clinically when neurotoxicity and diabetes are also present. A major part of learning to use FK 506 in kidney transplantation has to do with balancing nephrotoxicity with underdosing, and graft biopsies are often necessary.

Neurotoxicity is manifested principally by tremors, paresthesias of the extremities and insomnia, although other symptoms have been seen.[86] The symptoms have often been used as markers for dosage reduction and have generally been reversible. Serious neurologic side effects, such as seizures or confusion, have been seen rarely.[87]

Diabetogenicity has been seen in about 15% of non-diabetics receiving FK 506 after kidney transplantation.[88] It is largely

reversible after reduction in FK 506 and prednisone dosage, although a few patients have remained chronically insulin dependent.

Based on the initial clinical reports, FK 506 appears to be a promising new agent in kidney transplantation. While it is not without side effects and requires experience to use effectively, it has the potential for offering improved outcomes after kidney transplantation with less long-term morbidity.

MIZORIBINE (BREDININ)

Mizoribine (MZB) is an agent developed in Japan that has been evaluated in several clinical trials there. It has been used for over 10 years in clinical renal transplantation. Although initial studies employed it alone with steroids,[89] instead of azathioprine, in most of the recent studies, it has been used adjunctively with CsA and prednisone.[90-94] In trials comparing MZB with azathioprine, better or equivalent graft survival was seen with less bone marrow suppression and less infection. It thus appears to be safe and effective as a third agent in clinical transplantation.

As MZB levels tend to correlate inversely with creatinine clearance, its dosage needs to be adjusted downward in response to renal dysfunction.[95]

Given its efficacy and the number of years it has been used clinically, it is rather surprising that MZB has not yet come into

more widespread use. Hopefully, this will change in the next few years.

RS-61443

From the outset, RS-61443, a morpholinoethyl ester of mycophenolic acid, has been used adjunctively, with CsA and prednisone, in the treatment of renal transplant patients. There have been 2 trials thus far, a Phase 1 dosage trial and a rescue trial. Preliminary reports of both trials have been published,[69,96] and subsequent reports are in preparation. Dosages from 100 mg/d to 3500 mg/d have been employed with good tolerance and safety. Less rejection was seen at higher doses. The rescue trial looked at 20 cases of refractory rejection with conventional therapy, and found an 80% response to RS-61443 in doses of 2,000 - 3,500 mg/d. No evidence of nephrotoxicity, neurotoxicity, hepatotoxicity, or myelotoxicity was seen with this drug. There was one case of hemorrhagic gastritis, possibly related to the drug. In view of the relative safety and efficacy of the drug in preliminary studies, a multicenter randomized trial has been started, comparing CsA, RS-61443, and prednisone with CsA, azathioprine, and prednisone.

DEOXYSPERGUALIN (DSG)

Deoxyspergualin (DSG) is another immunosuppressive agent that has been developed and evaluated largely in Japan. It has been used in 2 clinical settings. As it is effective only if given intravenously, it has no role as a maintenance agent. Rather, it has been used in several studies to treat refractory rejection and has been shown to be effective both alone and when given with steroids.[97-100] The response rate ranged from 75 - 87%, and was over 90% when DSG and steroids were given together.

On the basis of the successful rescue trials, DSG was used for induction therapy, in combination with CsA-based regimens.[101-103] DSG induction has been successful in a variety of cases, - cadaveric, living-related, ABO-incompatible, and highly sensitized patients. Although the total number of cases is small, less than 40 to date, excellent patient and graft survival have been reported, as well as less rejection and nephrotoxicity. Essentially all of this clinical work has been in kidney transplant recipients in Japan, although there is one report from Sweden of the successful use of DSG to rescue a rejecting liver allograft.[104]

Side effects of DSG include reversible leukopenia; facial numbness or warmth, nausea, and anorexia have been noted as symptoms in a minority of patients, but have not been severe.

RAPAMYCIN AND BREQUINAR

To date, clinical reports on either rapamycin or brequinar have not yet appeared in the transplantation literature, although there is apparently a Phase I study of rapamycin in progress.

In addition to these new immunosuppressant drugs, there are several investigational agents that have not yet been evaluated extensively in the laboratory and have not yet been studied clinically.

CONCLUSIONS

Recently developed immunosuppressive drugs with distinct molecular actions, such as FK 506, rapamycin, RS61443 and brequinar sodium hold considerable promise for development of new, effective, and less toxic anti-rejection therapies. Given the rapid progress in understanding the molecular events underlying signal transduction in lymphocytes, it is likely that even more sophisticated drugs will be introduced which will allow further refinement of pharmacological immunosuppression.

In the next several years, we should see an explosion of clinical trials that will undoubtedly change the face of clinical immunosuppression as it is practiced today. The agents described in this chapter will probably have major roles in these trials and will likely become part of the immunosuppressive armamentarium of the future.

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Table 1 Modes of action of new immunosuppressive drugs

INHIBITORS OF CYTOKINE SYNTHESIS	
CsA	
FK 506	
INHIBITOR OF CYTOKINE ACTION	
RAPAMYCIN	
INHIBITORS OF DNA SYNTHESIS	
MIZORIBINE	↓PURINE SYNTHESIS
MYCOPHENOLIC ACID (RS61443)	
BREQUINAR SODIUM	↓PYRIMIDINE SYNTHESIS
INHIBITOR OF CELL MATURATION	
DEOXYSPERGUALIN	

Table 2 Comparison of the antilymphocytic activities of FK 506 and CsA in vitro

Response suppressed	Species	Conc. (nM)[a]	
		FK 506	CsA
IL-2, IL-3 and IFN-γ production	Mouse	0.1-0.3	3-32
IL-2, IL-3, IL-4, GM-CSF, TNF-α IFN-γ gene expression (IL-2 production)	Human	0.1-0.3	5-10
IL-2 receptor α chain; transferrin receptor expression	Human	0.1	10
MLR; generation of cytotoxic T cells	Mouse	0.2-0.3	24-27
	Human	0.2-0.3	20

[a] Concentration causing 50% inhibition

Table 3 Effects of CsA, FK 506 and Rapamycin on lymphocyte activation and proliferation

	CsA	FK 506	Rapa
Main binding protein	cyclophilin A	FKBP-12	FKBP-12
Effective concn.	μM	nM	nM
Reversibility	++	Ý	Ý
Inhibition of IL-2 synthesis	++	++	-
Inhibition of IL-2R expression	+	+	-
Inhibition of responses to:			
anti-CD3	++	++	++
TPA	-	-	++
TPA + anti-CD28	-	-	++
LPS	-	-	++
IL-2	-	-	++
Cell cycle stage inhibited	early G_0	early G_0	late G_1

TPA = 12-O-tetradecanoylphorbol-13-acetate

LPS = bacterial lipopolysaccharide

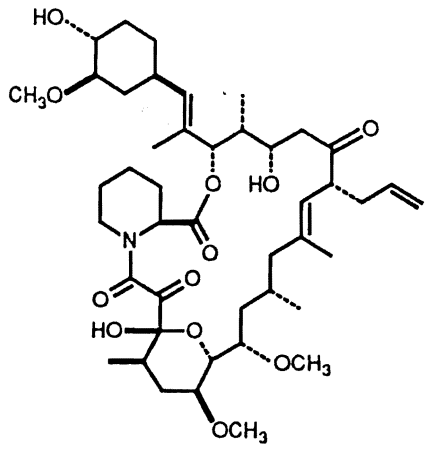
FKBP= FK 506 binding protein

Table 4 Actuarial one-year survival and biochemical parameters in FK 506-treated kidney transplant patients

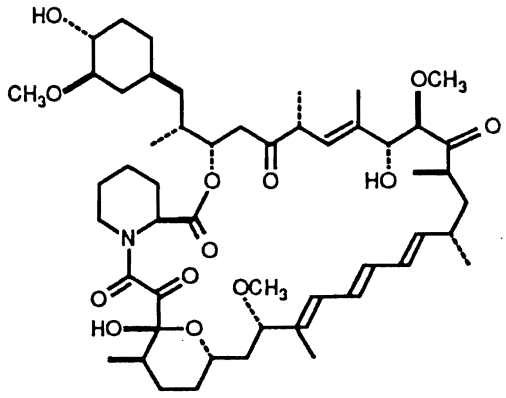
	CyA	FK 506	Total
Patient survival			
All	94%	90%	92%
Graft survival			
All	77%	74%	76%
Cadaveric	75%	73%	74%
Living donor	89%	100%	96%
Creatinine	1.9Ý1.7**	2.1 Ý 1.2	
BUN	31.0 Ý 17	36.0 Ý 22	
Uric Acid	7.2 Ý 2.6	8.1 Ý 2.4	
Cholesterol*	236 Ý 59	187 Ý 51	

*P < .0001;all others P = NS.

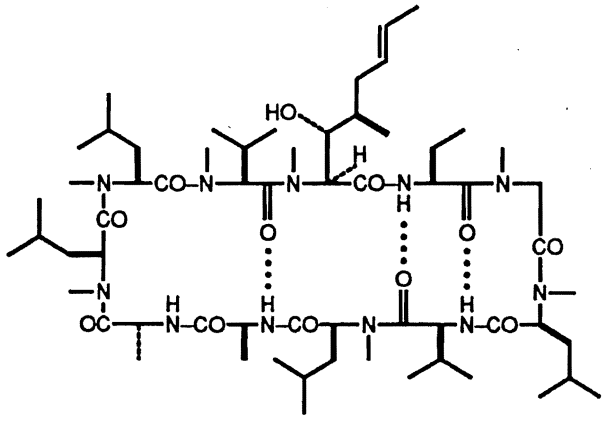
**All biochemical values are in mg/dL.
For further details see text and Shapiro etal[77]



FK 506



RAPAMYCIN



CYCLOSPORIN A

Fig. 1

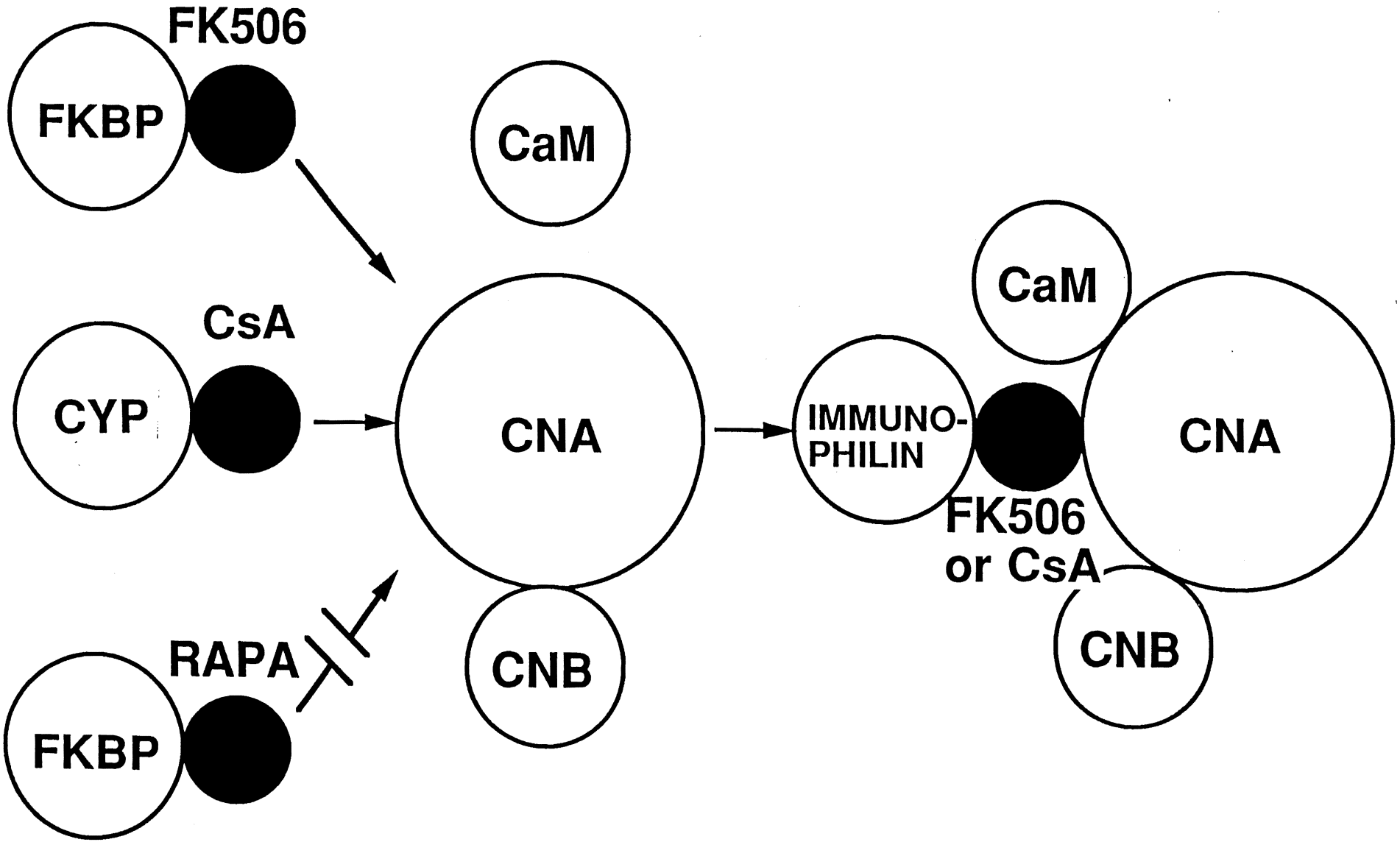

FK506
FKBP
CaM
CsA
CYP
CNA
IMMUNO-
PHILIN
CaM
CNA
FK506
or CsA
CNB
RAPA
FKBP
CNB

Fig. 2

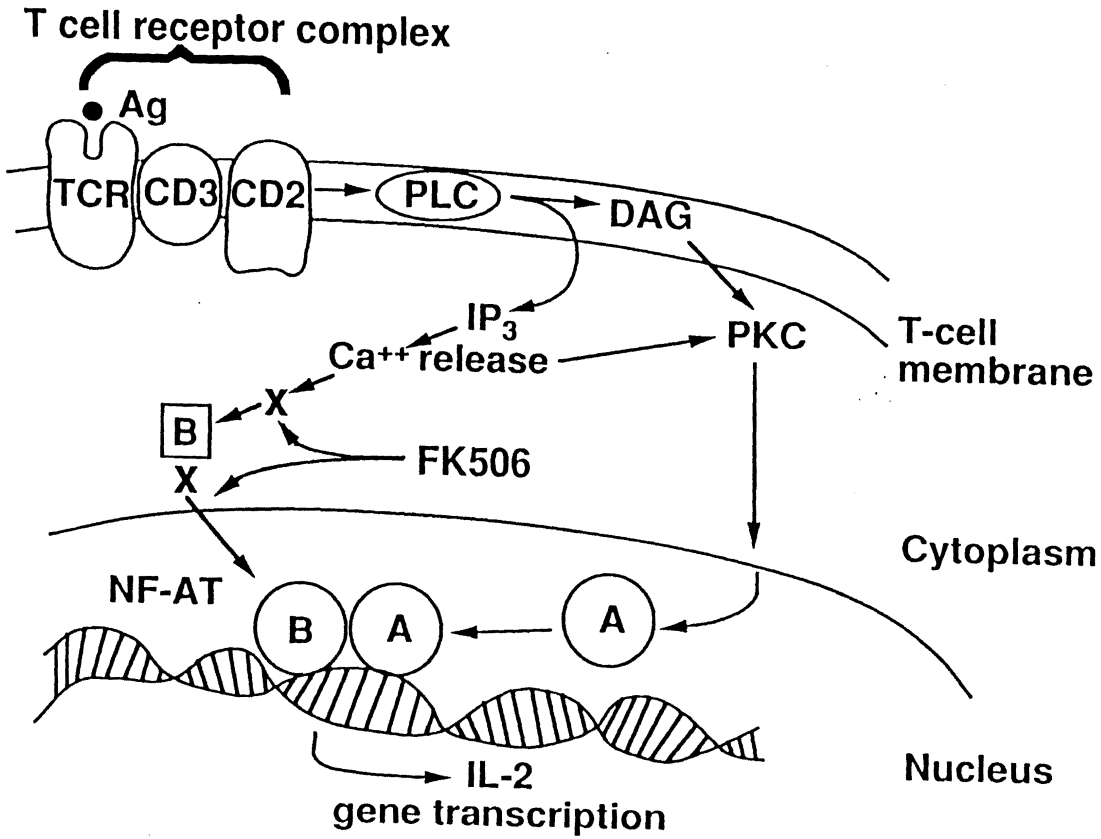

T cell receptor complex
Ag
TCR
CD3
CD2
PLC
DAG
IP3
Ca++ release
PKC
T-cell membrane
X
B
X
FK506
Cytoplasm
NF-AT
B
A
A
IL-2
gene transcription
Nucleus

Fig. 3

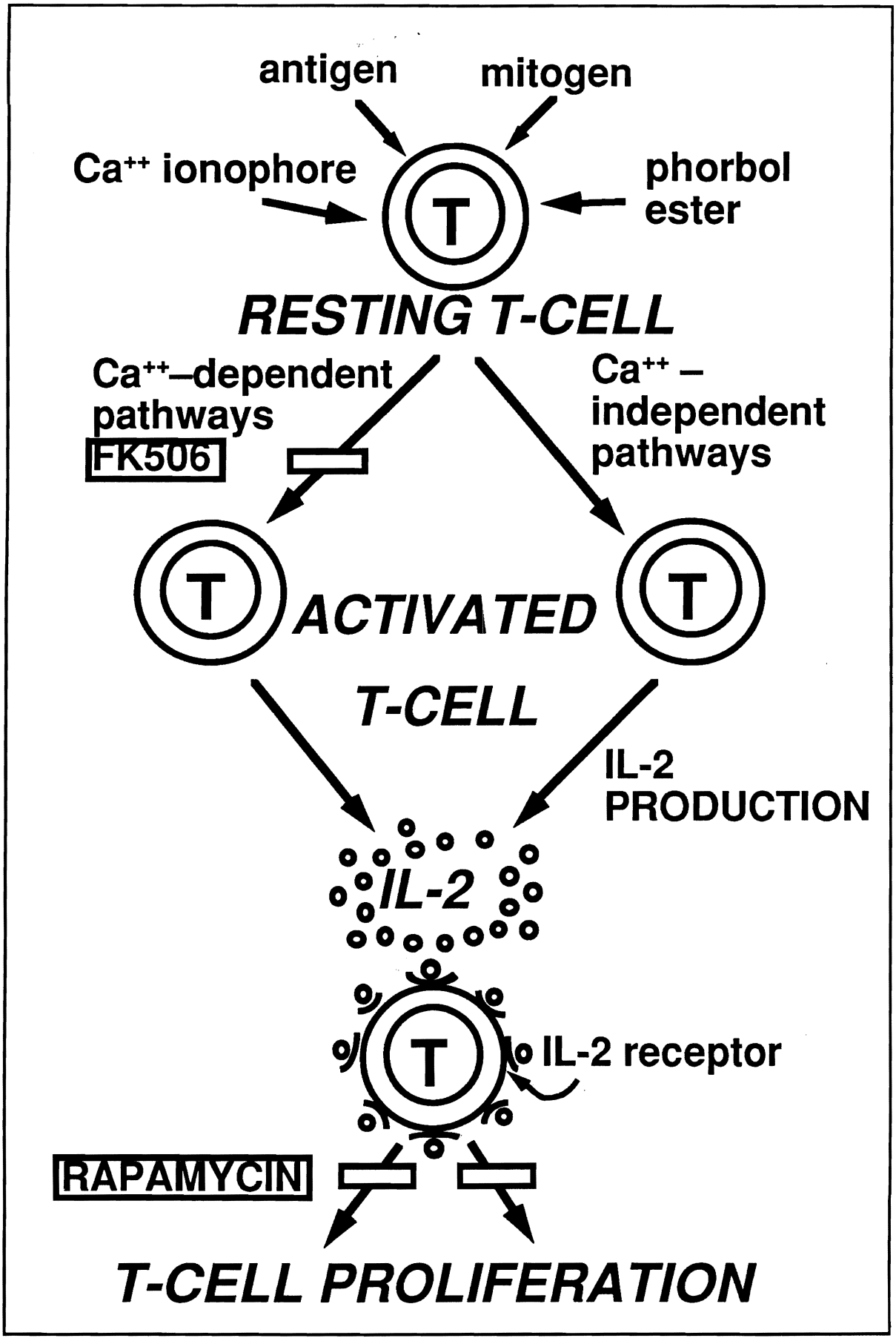
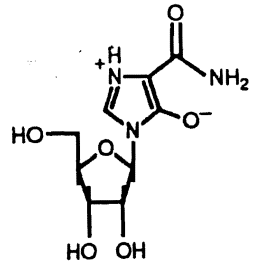
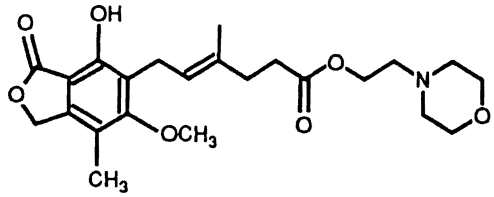

antigen
mitogen
Ca++ ionophore
phorbol ester
T
RESTING T-CELL
Ca++–dependent pathways
FK506
Ca++ – independent pathways
T
ACTIVATED T-CELL
T
IL-2 PRODUCTION
IL-2
T
IL-2 receptor
RAPAMYCIN
T-CELL PROLIFERATION

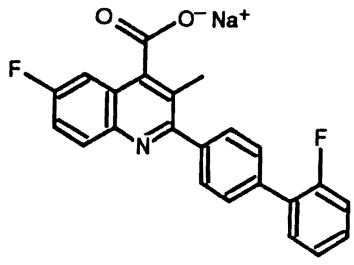
Fig. 4



MIZORIBINE

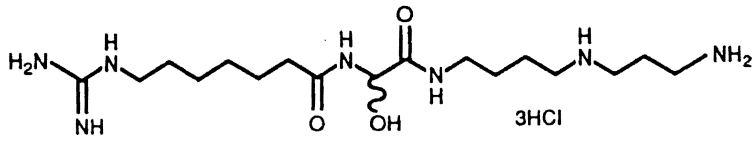


MYCOPHENOLIC ACID MORPHOLINOETHYL ESTER (RS-61443)



BREQUINAR SODIUM

Fig. 5



± DEOXYSPERGUALIN

Fig. 6