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NEW TRENDS IN IMMUNOSUPPRESSION

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Summary

The goal of immunosuppression in solid organ transplantation is to blunt the immune response of the patient to the allograft, while maintaining sufficient resistance to avoid opportunistic infections and malignancy. Despite progress in this field, rejection processes, particularly of the chronic form, remain an important cause of morbidity and graft loss. This review discusses the advances in drug development and pharmacology as well as in immunobiology, which are likely to lead to more potent, effective and selective regimens to improve the therapeutic efficacy and overcome the range of adverse side effects now plaguing the transplant enterprise. The future era of transplantation is likely to focus on receptor or cytosolic enzyme targets more specifically represented on or in lymphocytes as opposed to other cells or tissues. Four current targets of this strategy are: the chemokine receptor-7 surface marker that mediates lymphocyte affinity for specific types of high endothelial

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venules; Zap-70, a signaling enzyme associated with T cell antigen receptors (signal 1); plasma membrane proteins mediating costimulation (signal 2); and antagonists of Janus kinase 3 (Jak3), an enzyme transducing cytokine signals from the cell surface to the interior (signal 3).

Introduction

Following the observation that cell proliferation plays an essential role in immune responses, initial successful efforts with chemical immunosuppression began in 1960 utilizing the nucleoside antagonist 6-mercaptopurine (or its imidazole analog azathioprine). This strategy replaced the use of total body irradiation, a procedure not infrequently accompanied by excessive bone marrow suppression with emergence of serious infections (1-5). During the past 20 years a variety of other chemical antiproliferative agents have been tested without overcoming the hazards of azathioprine, including the alkylating agents cyclophosphamide, nitrogen mustard, chlorambucil and actinomycin-D (3, 6-8). While more potent antiproliferative agents have been recently introduced, such as mycophenolate mofetil (CellCept®; Roche Bioscience), or are poised for future drug development (brequinar and leflunomide), bone marrow suppression and emergence of viral infections remain significant hazards in addition to a pleiotropic array of toxic effects. The second strategy to be exploited for immunosuppression was T-cell depletion, since these elements were demonstrated to be important vectors of the immune response. Initially, polyclonal antilymphocytic globulins (pALGs) and, subsequently, anti-T cell monoclonal antibodies (MAbs) (9-12) were introduced to deplete or modulate alloresistance. Because the use of these agents is beclouded by a variety of problems owing to the intense immunosuppression, their therapeutic courses are limited to about 2 weeks. Despite this restriction, the risks of infection as well as lymphoma are significantly augmented. Less risky, albeit low potency antibodies directed against the alpha chain of the interleukin-2 receptor (IL-2R) have been recently introduced for therapy in the immediate posttransplant period.

The present era of immunosuppression was heralded by the discovery of the immunosuppressive activity of cyclosporine by Borel and its application in the clinical arena by Calne (13, 14). The selective effect of the drug on the production of cytokines not only reduced the acute rejection rates suffered by patients with solid organ transplants, but also tended to spare nonspecific host resistance. While tacrolimus (Prograf®; Fujisawa) proffered an even more potent (and toxic) mechanistic analog of cyclosporine, the next major advance in clinical immunosuppression was the discovery by Sehgal and subsequent clinical introduction of sirolimus (Rapamune®; Wyeth-Ayerst), which was recently approved by the United States Food and Drug Administration for use in renal transplant recipients (15, 16). This generation of agents disrupts the cytokine paradigm of alloimmunity: namely, production (cyclosporine, tacrolimus), reception by surface molecules (the MAbs against anti-CD25) and signal transduction (sirolimus). However, although these agents are selective for adaptive as opposed to nonspecific host resistance, they may produce significant collateral comorbidity in the organ systems of transplant recipients. Future progress in immunosuppression may depend upon development of agents directed against targets specific to T and B lymphocytes as opposed to other tissue cells. Lymphocyte recruitment at and diapedesis into the graft, the first step in direct antigen recognition, accentuates the ischemia and reperfusion injuries consequent to organ retrieval and transplantation. This cascade may be inhibited by selectin antagonists to block rolling, intercellular adhesion molecule-1 (ICAM-1) antisense oligodeoxynucleotides (oligos) to interrupt tethering and FTY720 to prevent diapedesis in response to tissue chemokines. Costimulation pathways, which are necessary for amplification of the antigen signal to produce full activation of the T-cell markers mediating response to generate cytokines in response to immune stimuli, represent an emerging target of pharmacotherapy with MAbs or receptor conjugates. The ultimate goal is specific inhibition of the antidonor response, leaving all other adaptive immune functions intact – transplantation tolerance. This strategy may overcome, or at least minimize, the need for chronic administration of immunosuppressive agents (Table I).

In the interim, until clinically relevant tolerance strategies are implemented, the current approaches to immunosuppressive therapy will exploit combinations of agents seeking to minimize the toxicity of each drug by synergistic drug interactions. This article will focus on the mechanisms of action, efficacy and toxicity of agents both in clinical use and in development, indicating the rationale for various combinations and the strategies to select

Table I: Paradigms of immunosuppressive therapy.

Paradigm	Agent
Proliferation	Azathioprine, MMF, BQR, leflunomide
Depletion/Modulation	Anti-T cell pAbs and/or MAbs
Cytokine	Cyclosporine, tacrolimus, sirolimus, SDZ-RAD, anti-CD25 MAbs
Ischemia-reperfusion-migration	Anti-selectin MAbs/antagonists, anti-ICAM-1 oligos, FTY720
Costimulation	Anti-B7, anti-CD154 MAbs; CTLA-4 Ig receptor conjugates
Transplantation tolerance	MHC peptides, allochimeric molecules, gene therapy

Data derived from Kahan, B.D. *New strategies in immunosuppression: Oligonucleotide therapy and allochimeric transplantation antigens.* BioDrugs 1997, 8 (Suppl. 1): 19-22. Used with permission.

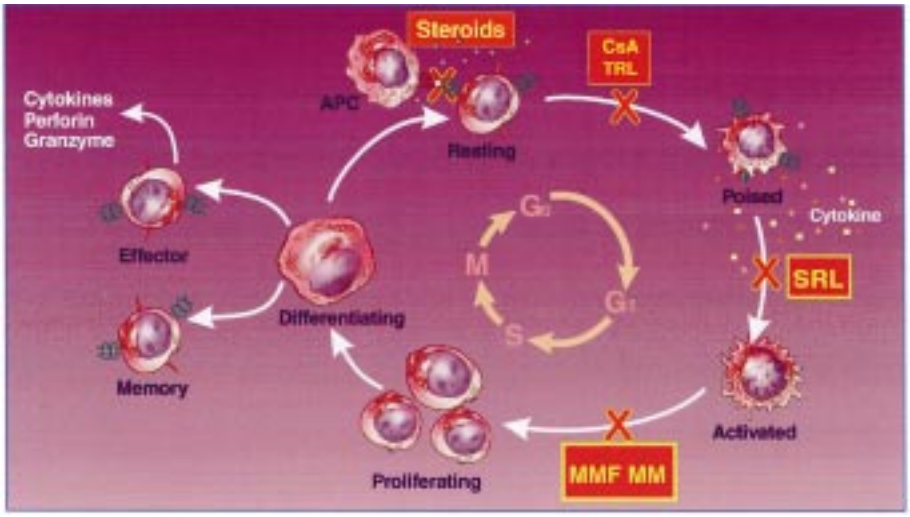

Fig. 1. The site of action of steroids (and other agents) in the evolution of the immune response.

drug doses during induction, antirejection and long-term maintenance phases.

Inhibition of Antigen Recognition: Corticosteroids

Corticosteroids were not only one of the first pharmacological agents used in transplant immunosuppression but also remain a cornerstone of most regimens despite their many side effects (17-21). Figure 1 shows the site of action of steroids (and other agents) in the evolution of the immune response. Only a small fraction of corticosteroids remains free in the plasma and not protein bound. Upon entry from this compartment into cells, steroids bind to cytosolic receptors that are present in the majority of mammalian cells, acting to upregulate or downregulate the activities of genes that bear specific DNA sequences (corticoid responsive elements). In lymphocytes, steroids seem to dampen the generation of activation protein-1 and inhibitory kinase kappa, thereby downregulating expression of genes encoding proinflammatory cytokines, including the costimulatory factors IL-1 and IL-6, and the proinflammatory molecules platelet activating factor, prostaglandins, leukotrienes and tumor necrosis factor-α (TNF-α) (22). Furthermore, steroids dampen chemotactic responses of oxidative free radical generation by expression of adhesion molecules on (23), generation of chemoattractants by and expression of cytotoxic activities of antigen presenting cells (APCs) (24, 25). In addition, steroids stabilize cell membranes from injury in toxic circumstances (26). Finally, bolus administration of high doses of steroids promotes emigration from the circulation of lymphocytes, thereby decreasing their availability to diapedese into the graft. Steroids thereby interfere with initial direct alloantigen recognition.

Four corticosteroid compounds are most often used in clinical transplantation – hydrocortisone, prednisone, prednisolone and methylprednisolone – because they display a high degree of antiinflammatory potency relative to their mineralocorticoid activity to cause sodium and water retention. Steroids are absorbed from the gastrointestinal tract rapidly and with high bioavailability (approximately 80%). Since steroids are primarily biotransformed by the liver through reduction and conjugation, changes in hepatic function may influence their elimination (27). Steroids are administered not only for long-term immunosuppression but also in high doses as the first line of therapy to control rejection responses as described by Bell in 1971 (21).

The diverse side effects of corticosteroids may be both serious and life threatening. Although the adverse reactions usually subside with dose reduction during maintenance therapy (28, 29), many effects have long-term, dose-independent consequences. The most serious complication is interference with APC as well as monocyte, macrophage and granulocyte function, leading to paresis of nonspecific host resistance and potential emergence of life-threatening bacterial, viral or fungal infections. A second set of adverse reactions are the metabolic changes contributing to hypertension, sodium retention, edema, weight gain, centripetal obesity, gastritis, peptic ulcer disease, glucose intolerance/diabetes mellitus, hyperlipidemia, osteoporosis, aseptic bone necrosis, peripheral neuropathy, psychosis, personality changes, pseudotumor cerebri, dermatological effects, cataracts, glaucoma and growth retardation in children. To minimize these side effects, therapeutic regimens seek to either avoid or markedly truncate the length of the steroid maintenance regimen. Although the latter strategy has been demonstrated to significantly reduce hypertensive and lipogenic toxicities (30), virtually every study shows an increased risk of allograft rejection (31).

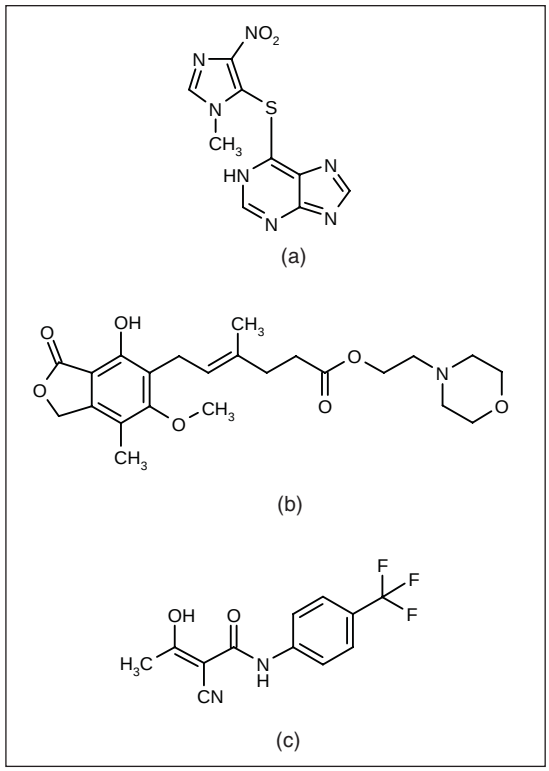

Fig. 2. The chemical structure of azathioprine (a), mycophenolate mofetil (b) and A-771726, the active metabolite of leflunomide (c).

Proliferation Paradigm

Azathioprine

Azathioprine, an imidazole derivative of 6-mercaptopurine, is a purine analog (Fig. 2a) that acts as an antimetabolite to inhibit nucleic acid synthesis via both *de novo* and salvage pathways (32). Rapidly dividing cells, including not only T- and B-cell lymphocytes but also, for example, gut endothelium and bone marrow elements, are most susceptible to the drug's antiproliferative effects. In addition, it has been suggested that the imidazole residue of azathioprine alkylates thiol groups on T-cell surface membranes block antigen recognition (33).

After oral administration, azathioprine is rapidly absorbed from the gastrointestinal tract (bioavailability approx. 50%) and cleared from the circulation by conversion to 6-mercaptopurine. The two main metabolic pathways of further degradation are oxidization by xanthine oxidase and methylation to several active derivatives, yielding 6-thiouric acid as the final biotransformation product (34).

Myelosuppression, particularly leukopenia, is the most common and serious side effect of azathioprine therapy (35). During treatment, the white blood cell count must be monitored; when it is less than 3000/l(x10^6) the dose should be reduced or

the drug withheld. Xanthine oxidase inhibitors such as allopurinol exaggerate bone marrow suppression and must be administered cautiously to patients receiving azathioprine. Azathioprine-induced hepatotoxicity is believed to result from endothelial damage, which disrupts liver blood flow and may cause veno-occlusive disease (36). Pancreatitis, the mechanism of which is unknown, has been reported infrequently. Febrile reactions, presumably representing an allergic reaction to the drug, demand cessation of treatment. Other toxicities relate to its antimitotic effects, including alopecia, oral ulcers, nausea, vomiting, diarrhea, anorexia and esophagitis. The increased incidence of malignancies with azathioprine tends to be less than that observed with other immunosuppressants, probably owing to its modest relative potency (35, 37).

Mycophenolate mofetil

Mycophenolate mofetil is a synthetic analog (Fig. 2b) of mycophenolic acid, a compound that is produced by several *Penicillium* species. The mycophenolate mofetil prodrug is hydrolyzed by widespread esterases to the active substance mycophenolic acid and biotransformed in the liver to an inactive glucuronide. As opposed to the varied sites and competitive action of azathioprine as an inhibitor of the nucleic acid synthesis pathways, mycophenolic acid is a selective, reversible, non-competitive antagonist of inosine monophosphate dehydrogenase activity (38, 39). To a greater extent than other cells, which can effectively use the salvage pathway, T and B lymphocytes are dependent upon *de novo* nucleic acid synthesis and have been claimed to be especially susceptible to mycophenolic acid. However, the fact that one of the primary toxicities of mycophenolate mofetil therapy is bone marrow depression, particularly leukopenia, suggests that the drug is not an exclusive inhibitor of T and B cells. Furthermore, the most common and dose-limiting toxicity is gastrointestinal irritation, including diarrhea, abdominal cramps, nausea and vomiting, as well as hemorrhagic gastritis and pancreatitis, which relate, at least in part, to the present commercial formulation of mycophenolate mofetil since they occur far more infrequently with the new ERL080A preparation (Novartis) (40). These toxic effects are compounded by an increased incidence of opportunistic infections, particularly due to cytomegalovirus, and associated lymphomas compared with azathioprine therapy.

Although the immunosuppressive effects of mycophenolic acid have been known for more than a century, interest was reawakened by Mitsui and Suzuki in 1969, who rediscovered its immunosuppressive properties (41). To increase the low oral bioavailability of mycophenolic acid, a mofetil group was added to the compound. Following seminal immunologic experiments by Allison and later clinical trials by Sollinger, multicenter trials showed a benefit of mycophenolate mofetil over azathioprine to reduce the incidence of acute rejection episodes among renal allograft recipients under treatment with a concomitant regimen of cyclosporine and steroids (40, 42).

The drug has been approved as an adjunctive agent for rejection prophylaxis, but the data on its efficacy for treatment of acute rejection episodes resistant to high-dose steroids, antilymphocyte globulin or OKT3 therapy are not yet conclusive. While mycophenolate mofetil was claimed to prevent the emergence of chronic rejection in animal studies, it has not been proven to achieve a similar effect in large trials in human renal transplantation (43-45).

Leflunomide

Upon metabolism by opening of its isoxazole ring, the heterocyclic compound leflunomide is degraded to an active, major metabolite A-771726 (Fig. 2c). While leflunomide was initially developed as an antiarthritic drug, it was later shown to display bifunctional immunosuppressive properties (46, 47). At high concentrations, leflunomide blocks the activities of protein tyrosine kinases and at low concentrations, dihydroorotate dehydrogenase. The latter action seems to be overcome by concomitant treatment with the end product of pyrimidine metabolism – uridine. In animal models, leflunomide modestly prolongs rat cardiac allograft survival and displays an additive interaction with cyclosporine (48, 49). In combination with cyclosporine and mycophenolate mofetil, leflunomide has been reported to prolong the survival of islet allografts or xenografts more potently than it affects the outcome of solid organ transplants (50, 51).

The side effects of leflunomide, including anemia, diarrhea, as well as histopathologic changes in the small bowel and liver, restrict its clinical usage. While leflunomide itself is predominantly being evaluated for the treatment of autoimmune diseases, such as rheumatoid arthritis, structural malononitrilamides (MNA 715 and 279) are currently under investigation in experimental

animal models of solid organ transplantation (52, 53).

Depletion Paradigm

Based upon the pioneering work of Woodruff, which demonstrated that heterologous antilymphocyte sera prolonged skin transplant survival in rats, Starzl used equine pALG to treat allograft rejection in 1967 (9, 10, 54). Later, Najarian *et al.* introduced their use for induction therapy to provide intensified rejection prophylaxis, thereby affording a window of up to 2 weeks for recovery of renal function before the inception of immunosuppressive treatment with nephrotoxic agents such as cyclosporine and tacrolimus (11). Several recent studies and meta-analyses suggest potential benefits of pALG both for induction prophylaxis and for treatment of acute rejection episodes (55-61).

Following immunization of rabbits, horses or goats with human lymphoid cells and separation of the globulin fraction by ion-exchange chromatography of serum pools, antibodies against red blood cells and platelets are removed by immunoabsorption techniques to yield pALG. At present, the preparations available in the U.S. have been raised in rabbits (Thymoglobulin®; SangStat) or in horses (ATGAM®, Pharmacia & Upjohn). The usual doses are 1.5-2.0 or 10.0-30.0 mg/kg/day administered via a peripheral or central vein, respectively. Another rabbit preparation (Fresenius AG) is available outside the U.S. The rabbit products are more potent and toxic than the equine sera (62). The clinical surrogate for immune effects is enumeration of peripheral blood T cells; the target is a value of about 150/mm^3, in contrast to normal numbers of 1100-2100/mm^3.

The pALG produce lymphocyte depletion predominantly by opsonization of T lymphocytes via complement-dependent lysis. Because of the polyclonal nature of the preparation, there are multiple antibody components that bind to distinct antigenic epitopes, presenting multiple sites for complement activation.

The second generation of development of antibody therapy harnesses hybridoma techniques to prepare B-cell clones that produce a single antibody idiotype – MAbs. After immunization of mice against human lymphocytes, the clone is selected for commercial development if it displays reactivity against a surface structure specific for lymphoid cells. The B lymphocytes producing the desired antibody are fused with a myeloma cell to produce an immortalized hybridoma that is cloned and propagated indefinitely (63). The original clinical reagent, OKT3, is a MAb directed against the epsilon chain of the CD3 marker, which is an integral part of the T-cell antigen receptor complex. OKT3 is usually administered intravenously (i.v.) 5 mg/day for 10-14 days or up to 21 days. Within minutes of delivery of MAb, CD3+ markers are not detected on lymphoid cells. Although high doses of MAb may produce T-cell depletion, in clinical practice T cells continue to be present in the circulation, as documented by the presence of cells bearing CD2, CD4 or CD8, but not CD3, markers. This phenomenon is termed immunological "modulation". Reappearance of more than 10% of peripheral T cells displaying CD3 markers during OKT3 therapy suggests a treatment failure, usually due to production of anti-OKT3 antibodies (64), as detected in patient sera using an enzyme-linked immunosorbent assay.

Antilymphocyte antibodies may engender significant side effects. Flu-like syndromes, fever, chills, nausea, vomiting, diarrhea and headaches may develop as early as 1 hour after the first and, to a lesser extent, subsequent doses due to antibody-stimulated release of cytokines from T cells, namely, TNF-α, IL-2 and interferon-(IFN) γ. Severe reactions include dyspnea, tachycardia, arthralgia, hypotension/hypertension and, in rare instances, pulmonary edema, particularly in patients with fluid overload or congestive heart failure. Another serious complication is aseptic meningitis, which may be differentiated from an infectious etiology by the absence of leukocytes or monocytes upon microscopic examination of fluid derived by a spinal tap. In addition, leukopenia and thrombocytopenia may occur because of cross-reactivities on these circulating elements. The cytokine release syndrome may be dampened both by premedication with steroids, antipyretics and antihistamines, as well as by use of low initial MAb doses. However, over-immunosuppression with these products increases the risk and severity of opportunistic infections, particularly those caused by cytomegalovirus and by posttransplant lymphoproliferative disorders (65).

In contrast, two new MAbs recently approved in the U.S. are free of the toxicities produced by the cytokine release syndrome for several reasons. First, their antigenic target – the α chain (CD25) of IL-2R – is selectively expressed on activated, but not on naive, T cells. Second, in contrast to the long-signaling cytosolic tails of the β (CD122) and γ (CD132) transmembrane protein components of the IL-2R, the short α chain (CD25) does not trigger cytokine release. Third,

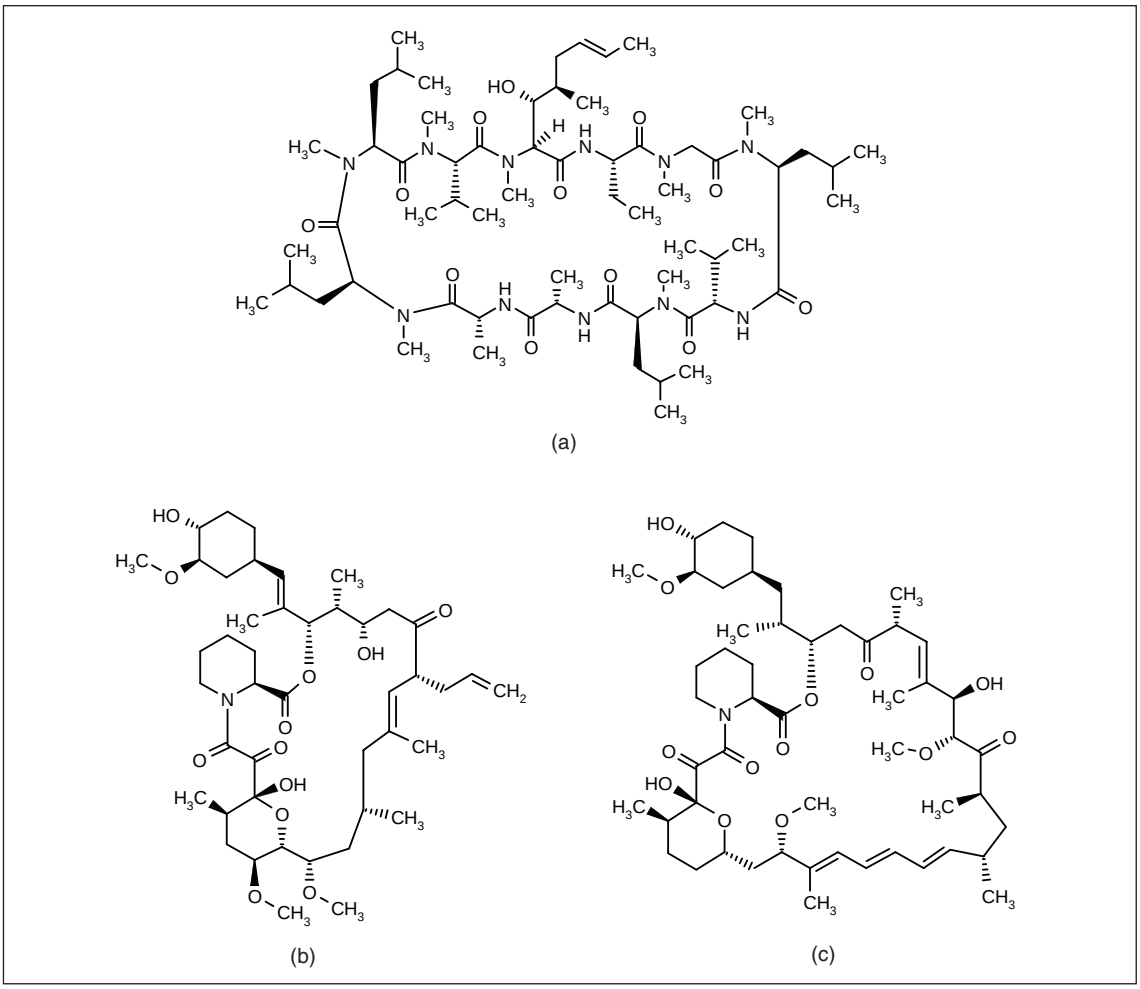

Fig. 3. The chemical structure of cyclosporine (a), tacrolimus (b) and sirolimus (c).

the reagents are chimeric (basiliximab) or humanized (daclizumab), and thus have a longer serum half-life and do not readily elicit neutralizing antibodies, problems associated with the pure xenogeneic polyclonal antibodies (pAbs) (66). Anti-IL-2R MAbs have been used mainly for induction therapy to reduce the occurrence of acute rejection episodes (67-69); they are not effective for rejection reversal owing to their modest potency.

Cytokine Paradigm

Cyclosporine

Cyclosporine, a cyclic fungal endecapeptide (Fig. 3a), acts primarily on T cells and to a lesser extent on B cells by blunting T-cell help (70). Hydrophobic cyclosporine molecules diffuse freely through the plasma membrane into the cytoplasm therein binding to a *cis-trans* peptidyl-prolyl isomerase, cyclophilin (71). Cyclosporine-cyclophilin complexes bind to catalytic units of calcium, calmodulin and calcineurins A and B, thereby blocking calcineurin phosphatase activity toward a variety of substrates, including the phosphorylated forms of nuclear factor of activated T cells, of nuclear factor κB and of c-Jun N-terminal kinase, all of which regulate the expression of T-cell growth promoting genes. In lymphocytes, cyclosporine blocks gene transcription and generation of mRNA for certain cytokines (IL-2, IL-3, IL-6,

IL-7, IFN-γ) (72, 73). In various nonlymphoid cells, calcineurin catalyzes dephosphorylation of other substrates, an effect that probably accounts for drug toxicities.

The original oral cyclosporine formulation (Sandimmune®; Novartis) requires the action of bile and succus entericus for saponification to permit absorption from the upper small intestine. The absorptive process shows frequently low and highly variable bioavailability (5-89%) because it is influenced by dietary composition, postoperative ileus, gastroparesis, diarrhea and cholestasis (74). Because of the importance of adequate early exposure to cyclosporine to avoid acute cellular rejection episodes, a microemulsion formulation (Neoral®; Novartis) was introduced; it shows higher bioavailability, less dependence on bile for absorption and lower intrapatient variability.

Cyclosporine is distributed extensively throughout the body. In blood, more than 50% of the drug is bound to erythrocytes, and the majority of the remainder in plasma (30-40%) is primarily bound to lipoprotein fractions, increased levels of which may reduce drug clearance rates (75). The biliary route is dominant over renal excretion (76). Cyclosporine is primarily metabolized in the liver via hydroxylation and demethylation reactions catalyzed by the cytochrome P450 system. Thus, cyclosporine metabolism is accelerated by the drugs that induce cytochrome – phenytoin, phenobarbital, valproic acid, carbamazepine and rifampin – and retarded by drugs that inhibit it – ketoconazole, erythromycin, diltiazem, verapamil and oral contraceptives.

Unfortunately, the use of cyclosporine is associated with a pleiotropic range of serious adverse effects. Nephrotoxicity is the most prominent and dose-limiting side effect (77). When the acute form of nephrotoxicity occurs immediately after transplant, the patient may experience oliguria/anuria, azotemia, hyperkalemia and renal tubular acidosis. The toxicity may reflect a rapid onset of afferent arteriolar vasoconstriction, which reduces blood flow and glomerular filtration rates, possibly due to enhanced local renin, angiotensin or endothelin release, to an altered prostaglandin balance or to enhanced sympathetic tone (78). Although this toxic reaction is usually reversible, it can markedly complicate the posttransplant course. A subacute and concentration-dependent form of nephrotoxicity is characterized by a tubular toxicity, as evidenced by hyperkalemia, hypomagnesemia and hyperuricemia. Another nephrotoxic problem that may occur early after transplant is hemolytic uremic syndrome, reflecting an idiosyncratic reaction to cyclosporine (79). Chronic nephrotoxicity is characterized by histological changes of vascular obliteration, interstitial fibrosis and tubular atrophy. This nephropathy, which is usually neither dose-dependent nor reversible, may contribute to renal allograft failure. Hypertension is a common side effect both in transplant and nontransplant patients treated with cyclosporine, due in part to vascular dysfunction and in part to sodium retention, which exacerbates the effects of steroids (80).

Adverse neurologic effects including tremors, headaches, paresthesias, confusion, hallucinations and seizures have become infrequent with wider appreciation of appropriate drug concentration monitoring. Similarly, hepatotoxicity, as evidenced by elevated aminotransferases and bilirubin, may reflect excess drug concentrations; however, this occurs rarely at present and is generally completely reversible. Metabolic side effects include hyperglycemia, which seems to be related to an islet cell toxicity, and hypomagnesemia. Of concern is hypercholesterolemia, which may result from drug-induced alterations in lipoprotein uptake by the liver. Cosmetic side effects, which also rarely present a clinical problem, include hirsutism and gingival hypertrophy. Finally, cyclosporine-treated patients have been reported to have an increased incidence of malignancies induced by viral infections, including herpes virus-related Kaposi's sarcoma and Epstein-Barr virus-induced lymphomas (81).

Tacrolimus

Tacrolimus, a macrocyclic lactone antibiotic (Fig. 3b), is a mechanistic analog of cyclosporine: namely, it also inhibits calcineurin activity (82). In contrast to cyclosporine, which binds to cyclophilin, tacrolimus binds to FK-506 binding protein-12 (FKBP-12), which also is a peptidyl-prolyl *cis-trans* isomerase. Because the two drugs share a common intracellular target, they display antagonistic interaction both *in vivo* and *in vitro*. The immunosuppressive activity of tacrolimus, first demonstrated by Ochiai, was confirmed thereafter in human liver, kidney, pancreas, heart and lung transplant recipients (15).

Despite its relatively bile-independent absorption, tacrolimus displays high interindividual variability of oral bioavailability (5-67%, mean value = 27%) (83). In peripheral blood, the drug is 75-99%

protein bound. It is distributed extensively in tissues throughout the body. Like cyclosporine, tacrolimus is primarily metabolized by the cytochrome system and its concentrations are affected by the same coadministrated drugs. Because the pharmacokinetics of tacrolimus show great intra- and interindividual variability, therapeutic drug level monitoring is essential to tailor treatment (84).

Clinical studies show that at least some of the toxicities of tacrolimus are similar to those displayed by cyclosporine (82, 84, 85). Tacrolimus causes a similar degree of nephrotoxicity while apparently producing less hypercholesterolemia. Although tacrolimus does not seem to produce hirsutism and gingival hyperplasia, it does cause more severe neurotoxicity, including headaches, tremors, confusion, psychosis, convulsions and seizures, than cyclosporine. Furthermore, tacrolimus produces a severe pancreatic islet β-cell toxicity that exacerbates diabetes mellitus, and tacrolimus therapy is beclouded by an increased risk of infections and malignancies, especially lymphomas (86).

Sirolimus

Sirolimus, a macrocyclic lactone (Fig. 3c), has a similar hemiketal structure masked α,β-diketone amide moiety like tacrolimus, but also has a unique triene segment (16, 87). The potential of sirolimus as an immunosuppressant was first reported by Sehgal *et al.* and later confirmed by Calne and Morris (16, 87, 88).

After binding to the immunophilin FKBP-12, sirlimus blocks G_1 cell cycle progression. In contrast to tacrolimus and cyclosporine, which inhibit G_0 to G_1 progression, sirolimus prevents progression from the G_1 to the S phase of the cell cycle by inhibiting the multifunctional kinase – mammalian target of rapamycin. This enzyme is a critical intermediate in IL-2 and other growth factor-mediated signal transduction processes. This inhibition blocks generation of the 70-KD S6 kinase necessary for ribosomal protein synthesis, prevents degradation of $p27^{kip1}$ necessary for cyclin generation and retinoblastoma protein hyperphosphorylation, and interrupts dissociation of the translation initiation factors eIF4A/B necessary for inception of ribosomal protein synthesis (89-92). The action of sirolimus to disrupt cytokine and growth factor signal transduction is not limited to cells of the immune system, but also affects fibroblastic, endothelial, bone marrow and smooth muscle cells, a set of drug effects that may prevent arterial smooth muscle cell proliferation, vascular disease and chronic rejection (93).

Although sirolimus is absorbed rapidly from the gastrointestinal tract, it shows a high degree of intra- and interindividual variability. The drug is metabolized by the cytochrome system, as are cyclosporine and tacrolimus. Although sirolimus may cause gastrointestinal irritation and transient bone marrow suppression, the most important side effect is hypertriglyceridemia (94, 95). Because the occurrence and severity of side effects correlates with the trough concentrations of the drug, therapeutic drug monitoring may avert or reduce the severity of these side effects (96). Sirolimus is not nephrotoxic in either psoriasis patients or renal transplant recipients treated with a regimen of azathioprine and prednisolone immunosuppression.

In experimental animal models, sirolimus has been shown to produce dose-dependent prolongation of the survival of skin, heart, kidney, small bowel and pancreaticoduodenal grafts (97, 98). In clinical trials, treatment with the drug as base therapy is associated with an acute rejection rate that is similar to that of cyclosporine (95, 99, 100). In contrast, patients treated with a combination of sirolimus and cyclosporine display acute rejection rates between 7-17%, suggesting a synergism possibly due to the complementary actions of the drugs. Furthermore, this enhanced potency seems to allow marked cyclosporine dose reduction, as well as early withdrawal of steroids.

Ischemia-Reperfusion-Migration Paradigm

A new target for therapeutic interaction is the initial series of events that occur upon revascularization of the allograft. The first step in the leukocyte adhesion process is rolling along the endothelium. Selectin antagonists can block this step. Tethering, the second step in the adhesion process, is mediated by cross-linking of ICAM-1 and its ligand lymphocyte function antigen-1. Interruption of the second step has been targeted by antisense oligos and by MAbs. Finally, the migration step may be blocked by the sphingosine analog FTY720, which alters lymphocyte trafficking (101).

Selectin antagonists

Selectins mediate the initial rolling phase of leukocyte recruitment. Three approaches to inhibit selectin function are presently undergoing evaluation: MAbs, ligand antagonists and activators of

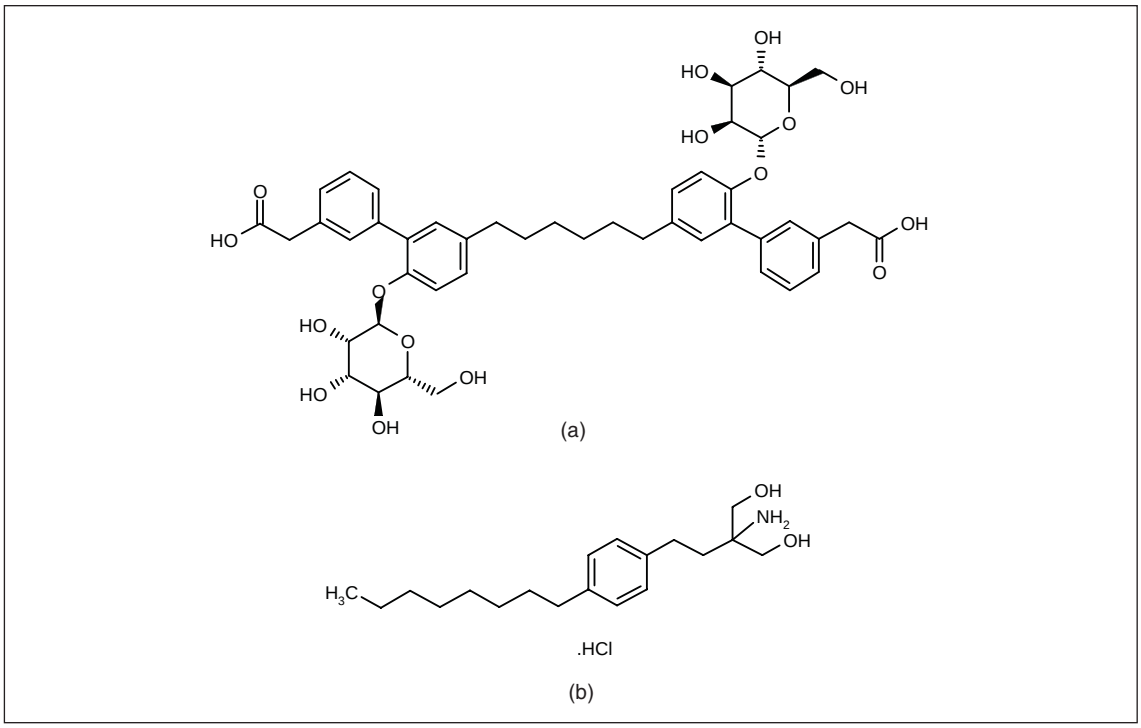

Fig. 4. The chemical structure of the selectin analog TBC-1269 (a), used with permission from Albany Molecular Research, Inc. and FTY720 (b).

receptor degradation (101). The novel immunoglobulin G MAb, KPL1, targets the human P-selectin glycoprotein ligand-1 (PSGL-1), which interacts with endothelial cell P-selectin (102). Pretreatment of murine liver allografts with KPL1 inhibited neutrophil infiltration (103), thereby mitigating both cold and warm ischemic injuries, as evidenced by dampened transaminase release and histologic damage (104). A second therapeutic approach administers sLe^x analogs: CY-1503 (105), which blocks P-, E- and L-selectin binding, has been shown in animal models to mitigate myocardial ischemia-reperfusion injuries (106). Another sLe^x analog, TBC-1269 (Fig. 4a), is a novel, small, nonoligosaccharide molecule that blocks selectins and dampens the ischemic injury. Although selectin inhibition *per se* will not provide full protection against leukocyte-induced tissue injury, this target may serve as a useful component of a multimodality strategy.

Antisense ICAM-1 oligos

ICAM-1 plays an important role both in the tethering of leukocytes to endothelium and as a coreceptor for the antigen-mediated signal 1 activation (107). Antisense oligos have been designed to hybridize with specific ICAM-1 mRNAs, thereby triggering RNase H, which digests the message selectively precluding protein translation of ICAM-1. Oligos are not only specific by virtue of their unique genetic code but also display a side effect profile distinctive from other immunosuppressive agents, namely, modest prolongation of partial thromboplastin time and minimal activation of complement components. Administration of anti-ICAM-1 oligos has been shown to prolong kidney transplant survival in animal models, whether treatment was confined to either the donor or the recipient (108).

Preliminary clinical trials suggest that there may be limitations to the use of oligos. First, their effect solely on ICAM-1 may not be sufficient by itself to block tethering; a cocktail of oligos may be necessary. Second, the highly negative charge of oligos restricts their penetration into cells, a problem that has been addressed using liposomes bearing encapsulated oligos or less highly charged peptidic or methoxy-, rather than

phosphorothioate, derivatives (109). An alternate delivery system is *ex vivo* transfection; cardiac allografts incubated with an anti-ICAM-1 antisense oligo under 3 atm pressure showed nuclear incorporation of the oligo, with consequent reduction in the expression of ICAM-1, as well as moderate immunosuppressive effects (110). A final limitation is the present requirement for i.v. oligo delivery.

FTY720

FTY720 is a synthetic analog (Fig. 4b) of the sphingosine-like compound myriocin. The mechanism of FTY720 action has only recently been elucidated. The original hypothesis that the drug induces apoptosis seems unlikely, rather it appears that FTY720 promotes lymphocyte homing to secondary lymphoid structures (111). Upon infusion of FTY720-treated fluorescent-labeled lymphocytes, the cells can be shown to sequester in peripheral and mesenteric lymph nodes as well as Peyer's patches, thereby diverting them from the graft (112). One hypothesis suggests that FTY720 upregulates expression of chemokine receptor 7 (CCR7) on lymphocytes, thereby increasing the affinity of these cells for high endothelial venules in secondary lymphoid structures, which are known to express the chemokines that bind CCR7. The action of chemokine CCR7 complexes rapidly upregulates integrin expression and cell adhesion.

In animal transplant models, FTY720 prolongs renal allograft survival and reduces the number of animals undergoing acute rejection episodes with 10- to 100-fold greater potency than cyclosporin (113-115). FTY720 acts synergistically with cyclosporine and/or sirolimus in rodent models, and displays at least an additive interaction with cyclosporine in canine and in subhuman primate transplant models (116, 117). In human studies, a single dose of drug causes a dose-dependent, rapidly reversible fall in the peripheral blood lymphocyte count that occurs at approximately 6-12 h and which recovers to the normal range by 48-72 h. Although no serious adverse events occurred among this cohort of patients, some subjects experienced bradycardia and exercise-induced hypoxia. In multiple-dose studies, a reduction in lymphocyte counts was observed with little evidence of serious toxicity. Phase II studies have just begun to explore the drug's clinical immunosuppressive activity.

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