Pediatr Nephrol (1993) 7: 567–573

Pediatric Nephrology

Invited review

New immunosuppressive agents for pediatric transplantation

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Received February 10, 1993; accepted February 26, 1993

Abstract. Pediatric transplantation has always been challenging for transplant surgeons. Although the higher immunoreactivity and the faster metabolism showed by this unique population when compared with adults requires a heavy immunosuppressive regimen, the possibility of disrupting the delicate balance of correct psychophysical development calls for a regimen of more selective and less toxic immunosuppressive drugs. In the past decade several new drugs have been investigated and some of them appear to be very promising, although pleiotropic toxicities have not yet been eliminated. An appropriate pharmacokinetic approach and the evaluation of synergistic multi-drug combinations by rigorous mathematical models would lead to highly selective immunosuppressive regimens which may result in virtually no toxicity.

Key words: Immunosuppressive agents – Transplantation – Cyclosporine

History of immunosuppression

Although the concept of transplantation is rooted in mythology [1], it has only been during the past half century that surgical experience and immunological knowledge have resulted in its routine clinical application. Despite the success of identical twin renal allografts performed by Murray in collaboration with Merrill et al. 1956 [2], not until 10 years later were transplants successful in children [3]. With better understanding of the immunobiology of rejection, increasing numbers of transplants were successfully engrafted into children during the subsequent decade [4, 5]. The obstacles to uniform success of transplants are, on the one hand, the more powerful immune responses of children and, on the other hand, the need of pediatric recipients for a minimally toxic immunosuppressive regimen in order to achieve the optimal rate of growth and development. The major advance that addressed these obstacles was the introduction of the fungal endecapeptide cyclosporine (CsA) which provided a relatively specific action on T cells [6], in contrast to the nonselective drugs, such as azathioprine (Aza) and corticosteroids, introduced in the clinical arena by Murray et al. [7] and Goodwin et al. [8], respectively, which act on elements of both specific and nonspecific resistance. A second advance in immunosuppressive therapy was the evolution of antilymphocyte preparations from polyclonal antilymphocyte sera, produced in horses or rabbits, which act against cell surface determinants present on human T and B lymphocytes, as well as on monocytes, granulocytes and platelets, to the murine monoclonal antibody OKT3 [9], which specifically recognizes distinctive surface markers on T cell plasma membranes. Although the integration of these agents into a variety of immunosuppressive regimens has improved the likelihood of transplant success, these regimens still produce considerable morbidity and do not effectively control rejection mechanisms leading to graft loss in the long term.

Agents and their actions

The current immunosuppressants can be divided into two groups based upon whether they act on a variety of tissues in a nonselective fashion [steroids, Aza, cyclophosphamide (Cy)], in addition to their actions on the immune system, or whether they specifically affect T and/or B cells by either inhibiting signal transduction pathways (CsA, FK506) or by recognizing selective T cell surface markers (polyclonal and monoclonal antibodies).

Steroids

Steroids act in the initial phase of the immune response by inhibiting the synthesis of co-stimulating lymphokines, in-

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cluding interleukin-1 (IL-1) [10] and IL-6 [11], by antigen-presenting cells. After steroids bind to them, intracytoplasmic heat-shock proteins translocate across the nuclear membrane to bind to specific DNA sites, thereby inhibiting gene transcription [12, 13]. In the pharmacological doses used for the treatment of rejection, steroids also drive lymphocytes out of the circulation, stabilize the membranes of cells under immune attack and interfere with the synthesis of other cytokines. Although steroids are potent immunosuppressive and anti-inflammatory agents, their pleiotropic side effects, including growth retardation, disfiguration, myopathy and osteopenia, make them a particularly hazardous class of drugs for pediatric patients.

Aza and related antipurine nucleoside drugs

Aza is a nitroimidazole derivative of the thiopurine drug 6-mercaptopurine (6-MP) [14] that was synthesized to protect the sulfhydryl group of 6-MP from in vivo methylation and subsequent oxidation. The immunosuppressive activity of Aza depends primarily on the formation of intracellular thiopurine ribonucleotides, which requires the conversion of 6-MP into thioinosinic acid by hypoxanthine guanine phosphoribosyltransferase (HGPRT) [15]. Thiopurines inhibit purine synthesis by competitive enzyme inhibition of both de novo and of salvage pathways, which involves the interconversion of purine bases [16]. Because Aza appears to more potently inhibit mixed lymphocyte reactions (MLR) than 6-MP [17, 18], additional mechanisms of action have been postulated, including the generation of unique drug metabolites by hepatic aldehyde oxidase via alternate catabolic pathways which bypass the generation of 6-MP. This catabolic cascade, which eventually generates thiouric acid, may include intermediates that display immunosuppressive properties [19]. A second hypothesis for the greater activity of Aza postulates that Aza causes cell surface action by alkylation of thiol groups, distinct from its antimitotic effect, and is based upon the capacity of Aza to inhibit formation of sheep erythrocyte rosettes around T cells [20, 21] and the MLR reactions by T cells from HGPRT-deficient patients [22]. Although Aza still tends to be used as a third agent with CsA and steroids, there is no evidence that it provides a major contribution. Furthermore, continued hepatic dysfunction, increased susceptibility to malignancy and troublesome papillomatosis becloud the long-term administration of Aza.

Newer antipurine nucleosides

The newer agents, Mizorbine (MZB) and RS-61443, act as noncompetitive inhibitors and are not incorporated into DNA, two advantages over Aza. MZB, a hydrophilic imidazole nucleoside antibiotic isolated from *Eupenicillum brefeldianum*, impairs T cell function by also depleting intracellular stores of guanosine monophosphate [23]. Widespread experience in Japan suggests that the drug is at least equally efficacious as, without producing the hepatic injury of, Aza [24]. RS-61443 is the semisynthetic morpholinoethyl ester of mycophenolic acid, produced by the fungus *Penicillin glaucum*, which also blocks de novo purine synthesis [25, 26]. The drug is now undergoing multicenter phase III investigations in renal allotransplantation, since preliminary work suggests that in doses of at least 2,500 mg it reduces the incidence of acute rejection episodes [27]. The long-term use of MZB or RS-61443 in pediatric patients is likely to produce adverse side effects akin to those of Aza.

Although it is thought that the drug combination MZB/CsA results in a better therapeutic effect than does Aza/CsA [24], MZB, mycophenolic acid (MPA) MPA and Aza have been shown in a rigorous mathematical model to exert only an additive effect when combined with CsA using in vitro assays [28].

Cyclophosphamide

Cy, synthesized in 1958, is an oxazaphosporine-substituted nitrogen mustard that acts as an alkylating agent [29]. The drug, originally developed as an anticancer compound, displays cytotoxic selectivity for neoplastic tissue with a better therapeutic index than other nitrogen mustard analogues [30]. After the loss of a chlorine atom and the formation of a positively charged intermediate, the drug irreversibly binds to nucleophilic sites, the most important of which are nucleic acids, thereby causing DNA cross-linking and cell death [31, 32]. Cy markedly depresses primary humoral responses in experimental animal models [33, 34] and inhibits human lymphocyte proliferation in response to the nonspecific mitogens phytohemagglutinin and pokeweed, as well as to specific antigens, purified protein derivative, candida and mumps [35–37]. Clinical transplantation has utilized Cy treatment in place of Aza in recipients suffering hepatic injury [38, 39] or for donor pretreatment in an attempt to reduce the content of passenger leukocytes in transplanted organs [40, 41]. Due to its high degree of toxicity and its narrow therapeutic window, Cy is rarely used for transplant immunosuppression today.

Selective anti-T cell agents polyclonal antilymphocyte preparations

Polyclonal antilymphocyte sera, which were first prepared by Metchinkoff at the beginning of the century [42], are obtained by immunizing animals with human lymphoblasts or thymocytes. The polyclonal reagents, regardless of their source (rabbit or equine), opsonize T cells, thereby leading to their removal from the circulation, reducing their numbers from 200–500/mm^3 to 50–150/mm^3. Additional mechanisms such as blindfolding, altered reactivity, or inactivation of residual T cells may explain the long-term immunodepression observed in some patients long after the completion of the therapeutic course. Although they represent the most powerful antirejection agents presently available, polyclonal sera produce a high degree of patient morbidity due to their unmasking of viral infections, particularly polyclonal lymphoproliferative diseases.

Monoclonal antibodies

Although monoclonal antibodies offer greater purity and molecular specificity, the widely used OKT3 (Orthoclone, Raritan, NJ), an IgG2a which binds the 17-kDa ε chain of the T lymphocyte pentapeptide surface antigen CD3 [43, 44], which transduces T cell receptor signals, is associated with serious toxicity and only moderate immunosuppressive activity.

Although high doses of OKT3 (10 mg) may opsonize circulating T lymphocytes, leading to their disappearance from the circulation [45], at the usual 5-mg doses it causes antigenic modulation of the CD3 complex from the T cell surface membrane without depletion. This modulation disrupts T cell recognition and signal transduction, thus producing immuno-incompetent T cells [46]. The limitations of OKT3 therapy include severe first-dose reactions due to the release of the lymphokine tumor necrosis factor and IL-2, adverse central nervous system effects, such as aseptic meningitis, induction of human antimouse antibodies, generally of the anti-idiotype variety, but not uncommonly of broader reactivity, a not infrequent incidence of rebound rejection episodes upon completion of the therapeutic course, susceptibility to opportunistic viral infections and after multiple courses, a high incidence of post-transplant lymphomas, particularly when used after previous administration of antilymphocyte serum.

Cyclosporine

CsA is a cyclic endecapeptide of molecular weight 1,202 Da which is extracted from the fungus imperfecti *Tolypocladium inflatum gams*. Of the 11 amino acids, 7 are *N*-methylated, thus conferring an overall hydrophobic character with a hydrophilic active site, including residues 11, 1, 2 and 3, towards the interior of the cyclic structure.

CsA inhibits T cell synthesis of IL-2, IL-3, interferon-γ (IFN-γ), IL-6 and IL-7, while its effects on IL-1 and IL-2 receptor generation remain controversial [47–49]. T helper and T cytotoxic cells are the primary targets of CsA; T suppressor cells are spared, probably due to their CsA-resistant activation cascade [50]. At the molecular level, CsA inhibits processes which depend on increased cytoplasmic calcium (Ca) burst. CsA binds to a cytoplasmic protein, cyclophilin (CYP), which displays *cis-trans*-peptidyl-prolyl-isomeraseactivity [51]. Binding to CYP causes the hydrophilic group at position 1 of CsA to evert from the interior to the exterior of the molecule [52], to presumably convert the pro-drug to an active moiety. Liu et al. [53] and Friedman and Weissman [54] suggested that CsA binding changes the substrate affinity of the isomerase. CsA-CYP complexes with Ca^{2+}, calmodulin and calcineurins (CaN) A and B, thereby generating a pentameric unit which inhibits CaN phosphatase activity and consequently cleavage of the phosphate from the nuclear factor of activated T cells, NF-AT, the first regulatory protein controlling the enhancer region of the IL-2 gene.

New CsA formulation and analogues

Numerous CsA analogues have been tested in an effort to improve the drug's therapeutic window [55]. Cyclosporin G is a naturally occurring analogue which bears a L-norvaline substituent at position 2. In several experimental models the drug displays immunosuppressive effects equal to CsA [56–58], but less nephrotoxicity in Wistar rats [59]. Initial clinical trials suggested that the drug can produce hepatotoxicity; however, recent trials suggest that this effect is dose related and that slightly better renal function is experienced than achieved with empirical, nonconcentration controlled regimens of CsA.

SDZ IMM-125 (Fig. 1) is a hydroxyethyl derivative of D-serine 8-cyclosporine which is only slightly more potent, but far less nephrotoxic, than CsA in animal and in vitro endothelial cell culture experimental models [60, 61]. Initial clinical trials were complicated by a failure to recognize the unique pharmacokinetics of the compound. The agent has two possible applications: as a substitute for CsA, in order to achieve better renal function, or as a drug to be used at high doses in order to achieve greater immunosuppression without the increased hazard of nephrotoxicity, the latter possibly being more important in pediatric practice. Some small children display low CsA absorption, less than 20% of the administered dose. Attempts to increase CsA bioavailability have involved the concomitant administration of vitamin E [62] or pancreatic enzyme preparations.

A recent advance is the dispersion of CsA in a glycofurol preconcentrate that forms a microemulsion. This formulation displays a twofold enhanced bioavailability over the existing liquid or gelcap preparations. Its absorption appears to be less dependent on bile than are the olive oil- and corn oil-based formulations.

Optimal immunosuppressive regimens for the pediatric population using available agents

Although numerous combinations of the above agents have been advocated by various groups, the majority (75%) of immunosuppressive protocols for pediatric kidney recipients utilize CsA, steroids and Aza, as documented by the 1989 report of the North American Pediatric Renal Transplant Cooperative Study [63]. Clearly, CsA is the linchpin of therapy, although the optimal regimen remains controversial.

The first issue of debate is the time of inception, some workers preferring immediate post-transplant treatment and others delaying treatment for as long as 1 week after transplantation. Since most investigators believe that pediatric patients as a group are at high risk for rejection, there is widespread use of antilymphocyte preparations for induction therapy lasting between 5 and 10 days. Furthermore, in pediatric transplant recipients, CsA displays pharmacological properties distinct from those found in adult transplant recipients [64], namely poorer absorption and much more rapid clearance rates. In addition, the pleiotropic toxicity of the drug demands appropriate adjustment of the management strategy in order to achieve thera-

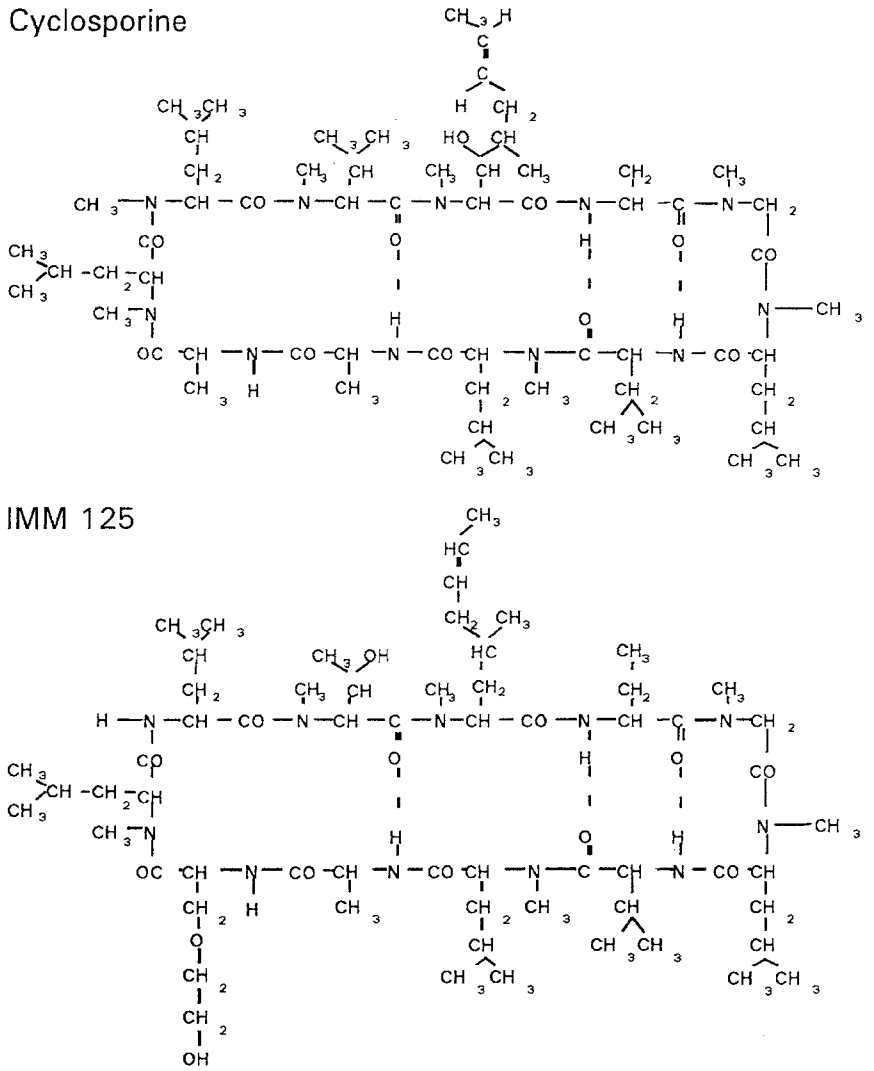

Fig. 1. New cyclosporine derivative SDZ IMM-125

peutic blood concentrations without nephrotoxicity, a particularly worrying complication in children, who depend upon adequate allograft function for optimal growth [65, 66].

In studies using empirical methods the median initial CsA dose was 9.6 mg/kg per day and the maintenance dose, at 60 days posttransplant, 5.7 mg/kg per day. In contrast, a pharmacokinetic strategy has been advocated in order to increase the efficacy of CsA management during the induction and maintenance phases of immunosuppression [67–70]. During the induction period, our individualized pharmacokinetic approach allows administration, by continuous intravenous infusion, of a dose determined by the results of a pretransplant pharmacokinetic study. This dose successfully achieves steady-state concentrations of 400 ng/ml. By using similar calculations based upon pre- and post-transplant test oral doses, the oral CsA dose is calculated to achieve a therapeutic target average concentration of 550 ng/ml per 24 h (area under the curve/unit) during the 1st post-transplant month. Monthly dosage adjustments then lower the target values, 500 ng/ml per 24 h at 2 months, 450 ng/ml per 24 h at 4 months and 400 ng/ml per 24 h at 6 months. The dosing interval is determined by the patient's rate of CsA clearance. Frequently children under 10 years of age require dosing three times a day, particularly when they are under simultaneous treatment with antiseizure medications.

The importance of an Aza component is directly related to the expertise and comfort of the physician with CsA dosing. Triple therapy is not uncommon during the first 6 months, although no controlled studies document its real benefit over dual-drug regimens. At 6 months, if the child has had a benign course, one may consider withdrawing one drug. If the child has the potential for further growth, it may be reasonable to withdraw steroids. If growth is not an important aspect, it may be wiser to withdraw Aza and thus to reduce the risk of hepatic injury and lymphoproliferative disorders. Clearly the withdrawal should be performed slowly, generally over a 3-month period, with careful follow-up for the next 6 months. In the case of living-related donor grafts, in vitro MLR and cell-mediated lympholysis assays prior to withdrawal may be useful to select and monitor candidates at low risk of rejection upon withdrawal of one agent.

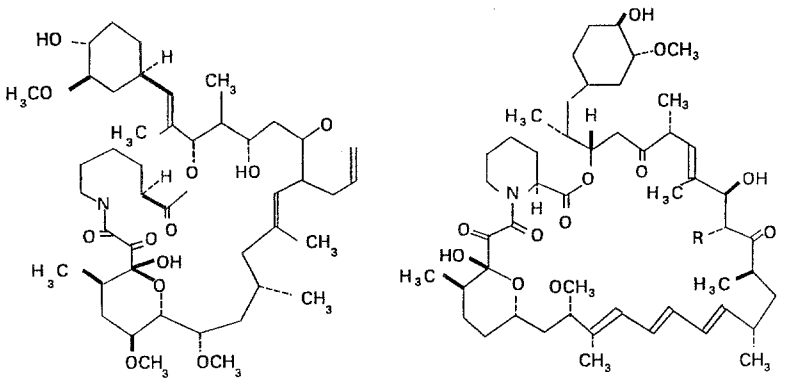

Fig. 2. New immunosuppressive macrolides rapamycin and FK506

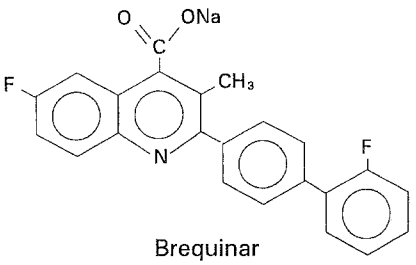

Fig. 3. Atomic structure of the new antiproliferative agent brequinar

New immunosuppressive agents

The optimal drugs would selectively interfere with the maturation and proliferation of allospecific T cells with minimal nonimmunological effects, however, even the newer, promising agents all display pleiotropic toxicities. Although children, particularly those under 10 years of age, may be less likely to display the nephrotoxic effects of CsA, their high level of immunoreactivity [71] appears to be associated with a CsA resistance. Thus there is special interest in the new immunosuppressive agents examined over the last few years: FK506, rapamycin (RAPA) and brequinar (BQR).

FK506

FK506, a polycyclic macrolide produced by a strain of *Streptomyces tsukubaensis* (Fig. 2), inhibits the production of IL-2, IL-3 and IFN-γ by T cells in vitro [72]. Like CsA, FK506 also perturbs Ca^{2+}, particularly intracellular pathways, but its effects upon T cell alloresponses are 10- to 100-fold more potent than those of CsA [73, 74]. Because FK506 and CsA appear to act on a common pathway, their immunosuppressive effects are antagonistic to each other [75]. A preliminary nonrandomized study of liver recipients showed that FK506 therapy displays greater neurotoxic, equivalent nephrotoxic, but possibly fewer hypertensive complications than does CsA [76]. FK506, has clearly failed to improve the results of kidney transplantation, because it does not provide better rejection prophylaxis than CsA [77]. While the results of randomized trials have not yet been reported, it is likely that the high degree of toxicity of the agent will only be overcome when the unreliable enzyme-linked immunoassay has been replaced by an accurate, sensitive measurement technique to guide concentration-controlled trials.

Rapamycin

RAPA (914.2 Da), a macrolide produced by the actinomycete *Streptomyces hygroscopicus*, is structurally related to FK506, except for a triene segment and a distinctive cyclohexane moiety (Fig. 2). Unlike CsA and FK506 which affect lymphokine synthesis, RAPA potently inhibits receptor-mediated cellular activation events [78] in the G1 phase of the cell cycle [79]. The drug does not interfere with the binding of radiolabelled IL-2 lymphokines to its IL-2 receptor, nor does it interfere with the phosphorylation of the 75-kDa beta-chain of the IL-2 receptor. In contrast, RAPA inhibits an enzyme p^{7056} kinase inase [80] involved in the G1 build-up and also blocks the endocytosis of the IL-2/IL-2 receptor complex. Because the site of action of RAPA is distal to that of CsA in lymphocyte activation [81], the two drugs show a synergistic interaction which may be of great clinical importance in permitting the reduction of the dose of each drug and thereby achieving effective immunosuppression with virtually no toxic side effects [82].

Brequinar

BQR is a synthetic difluoroquinoline carboxylic derivative which noncompetitively and reversibily inhibits mitochondrial dihydro-orotate dehydrogenase, the fourth enzyme in the de novo pyrimidine biosynthesis pathway [83, 84]. BRQ (Fig. 3) has two distinct effects: in addition to its effect as an antiproliferative agent which interferes with DNA synthesis, it also more importantly may show some effect on RNA synthesis and inhibit glycoprotein synthesis through a reduction in the generation of uridine diphosphate glycosylated compounds. The results of the rigorous median-effect analysis, show that there was not only an additive relationship between CsA and/or Aza, MZB or MPA, but a synergistic interaction between CsA

and/or RAPA and BQR, in both in vivo and in vitro systems [28]. Furthermore, permanent survival of heterotopic cardiac transplants is achieved in a rat model with modest doses of the triple combination CsA (0.5 ng/kg per day), RAPA (0.01 mg/kg per day) and BQR (2.0 mg/kg per day), demonstrating the possibility of a clinical synergistic strategy that achieves enhanced and more specific immunosuppression with minimal toxicity.

Acknowledgements. Supported by an award from the National Institute of Diabetes, Digestive and Kidney Diseases NIDDK 38 016-05.

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