New Immunosuppressive Reagents to Aid the Induction of Tolerance in Organ Graft Recipients

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Thirty years ago when the first immunosuppressive agents became available for clinical grafting,[1] the procedure was regarded with great skepticism by the medical profession as a pastime for ignorant surgeons with results that at best were poor. With the introduction of cyclosporin 15 years ago,[2] the results of organ transplantation improved, and for the first time it was possible to transplant lung and pancreas with a reasonable outcome. Now more than 200,000 organ grafts have been performed worldwide and the procedure is an important established part of therapeutic surgery.

Many of the currently used immunosuppressive reagents are the result of basic scientific advances, often following screening of natural products or deliberate synthesis for other purposes. Ideally, an immunosuppressive drug should be effective and nontoxic; it should manipulate the immune system in such a way that tolerance can be produced towards the graft in question without damaging the natural immune mechanisms of the body for protection against infection and neoplasia.

Anti-inflammatory corticosteroids are still used in organ transplantation and are the first choice for the treatment of acute cellular rejection in most clinics. Their action is rapid in terms of resolving inflammatory edema and the cell infiltrate. Nevertheless, steroids are not welcomed by patients for long-term treatment; especially unfortunate are the side effects in children and young women (change in facial characteristics and stunting of growth resulting in a "moon" face). Bone necrosis and collapse of the hip joint were features of high-dose steroids. Clearly there is room for improvement in the immunosuppressive treatment of organ graft recipients, and the ideal would be to induce donor organ-specific tolerance whereby all therapy could be stopped.

Donor Organ-Specific Tolerance

The therapeutic goal in transplantation is to create specific tolerance to a foreign graft, wherein all therapy can be stopped. "Near tolerance," requir-

ing minimal-maintenance immunosuppression, would also be of great benefit, and such operational tolerance has already been observed in long-term liver grafted patients, some of whom have had no immunosuppressive drug treatment for over 10 years.[3] Although *reliable* tolerogenic therapies have yet to be defined, progress in understanding the power and regulation of the immune response is guiding successes in this field.

1. Tolerance and Intolerance

Philosophically it may be argued that tolerance is a valuable asset. Certainly, at the physiological level, the physical state of immune self-tolerance is crucial, and immune dysfunction leading to self-*in*tolerance bears witness to the power of immune attack through the crippling consequences of autoimmune disease. A distortion of this scenario occurs where a foreign graft is placed into a normal recipient; here immune cells are induced to amplify and kill the graft. An even more dramatic outcome may follow engraftment of bone marrow, where the immune-competent foreign cells attack and kill the recipient.

Ideally, methods for the *selective* removal of immune responsiveness against donor graft antigens would allow the full potential value of transplantation to be realized. Moreover, patients with autoimmune disease might be cured by re-establishing immune self-tolerance to the autoantigens. Long sought after, the deliberate induction of specific tolerance is now possible in rodent models and promises to become realized clinically. This follows progress in understanding immune regulation. The lymphocyte is the unit of specific immunity, and herein extraordinary levels of self-control operate. Each lymphocyte response is dedicated to a single antigen, and the cell is essentially in a state of suspended animation until activated by this specific antigen. Then the lymphocyte divides rapidly to ensure numerical sufficiency in terms of combating and removing the source of the antigen. Thereafter, the antigen-specific population decreases to avoid cluttering up the lymphoid mass with redundancy, thereby retaining space for relevant future needs. Memory for previous encounters is maintained and permits accelerated responses to repeated antigen exposure which is manifested as accelerated rejection in recipients previously sensitized against donor-type antigens.

2. Therapeutic Strategies.

Within the history of transplantation to replace damaged vital organs, immunosuppressive therapies have evolved, first to inhibit cell division and thereby prevent the amplification step, and ultimately to selective induction of tolerance to the graft. Some of the more recent agents employed during this evolution are discussed below, together with the concept of generating donor organ-specific tolerance.

ANTIMETABOLITES

In the 1950s George Hutchings and Gertrude Elion[4] working at the Burroughs Wellcome Laboratory at Tuckahoe, New York, embarked on a program of synthesizing purine and pyrimidine analogues, the initial objective being to treat malignancies, especially leukemias and lymphomas. The rationale was for the synthetic analogue to be taken up by receptors; then, because of chemical differences from the parent nucleotide, the biological role would be inhibited and the proliferating cells would die.

One of the first effective agents to emerge from this program was 6-mercaptopurine (6-MP). Schwartz and Damashek[5] showed that this antileukemic agent would also interfere with antibody production in rabbits challenged with foreign antigens. The most intriguing aspect of their report was that the 6-MP given at the same time as soluble antigen during the inductive period of antibody synthesis completely inhibited production of antibodies, and this inhibition persisted in animals subsequently challenged in the absence of 6-MP. Thus a "drug-induced immunological tolerance" to foreign proteins antigen had been generated. Unfortunately, such clear production of tolerance cannot be achieved in animals with organ grafts, but *prolongation* of renal allograft function in dogs was demonstrated by use of 6-MP,[6] and subsequently improved results were obtained with a derivative of 6-MP, namely azathioprine.

Since the success of the work of Hitchings and Elion, many other workers have embarked on similar programs of synthesis of "fraudulent" metabolites, with the same objective in view. Some of these agents have been produced by chemical synthesis and others by fermentation of bacteria and fungi, with or without chemical modification.

Mizoribine (bredinin)

Mizoribine is a nucleoside isolated from a soil fungus which inhibits the growth of Candida albicans. It has been used in Japan since 1984, and it is believed to act by inhibiting inosine monophosphate dehydrogenase which stops the synthesis of guanine nucleotides.[7] The Japanese have found Mizoribine to be superior to azathioprine, but the drug has not been used extensively outside of Japan.[8] It can be toxic to the bone marrow, and some patients develop gastritis.

Mycophenolate Mofetil (RS-61443)

Mycophenolate mofetil is a new drug designed to inhibit lymphocytes[9] selectively and has been used successfully in the clinic.[10] It is now registered by the FDA in the United States for use as an immunosuppressive agent in organ transplantation. It is a fermentation product of certain penicillins and is thought to have a similar action to Mizoribine, inhibiting inosine monophosphate hydrogenase and interfering with both T- and B-cell proliferation. Experimentally it inhibits the proliferative arteriopathy of chronic rejection,[11] and this could be a valuable action of this compound if similar findings occur in the clinic. The most common side effect is diarrhea.

Brequinar Sodium

This is a synthetic quinoline carboxylic acid analogue which interferes with pyrimidine synthesis and was originally developed as an anti-cancer agent. It can cause myelodepletion. It has been found to be an effective agent in certain experimental xenograft models.[12]

SPECIFIC IMMUNOSUPPRESSANTS

Drug-Immunophilin Complexes

1. The immunophilins.

The immunophilins (recently reviewed[13]) are abundant cellular proteins which have prolyl cis-trans isomerase activity: these enzymes are normally involved in cellular house-

keeping functions including folding, transport, and stabilization of other cellular proteins. Importantly, certain immunophilins acquire novel, *highly potent* activities when complexed to specific drugs. The gain of function by the immunophilin/drug complex is immunosuppressive. Since the immunophilin-drug complexes cause complete immunosuppression when only approximately 1% of the total immunophilin pool is complexed, then the inhibition of prolyl isomerase activity per se is not the cause of immunosuppression. This is confirmed by drug analogues which bind and inhibit isomerase function but do not cause immunosuppression.

2. *Cyclosporin and FK506.*

Cyclosporin A was originally developed as a potential antifungal agent by Sandoz Pharmaceuticals (Basel, Switzerland), where Dr. Jean Borel discovered that it had specific immunosuppressive properties in vitro. Public presentation of this discovery led to the first use of cyclosporin A for clinical transplantation in Cambridge, U.K., in 1978.[14] It took another 12 years to unravel the mode of this drug's action; intriguingly, this appears to be identical to that of a more recently developed drug, FK506.[15,16]

Mode of action: The specific immunosuppressive drugs, Cyclosporin A, isolated from the fungus *Tolypocladium inflatums*, and FK506, from *Streptomyces tsukubaenis*, each act to prevent induction of the gene-encoding interleukin 2 (IL2) in T cells during antigenic stimulation. These drugs caused excitement among molecular biologists when it was discovered that, alone, each drug molecule is inactive, requiring complex formation with a cytoplasmic receptor (immunophilin, see above) to become immunosuppressive. Cyclosporin A is a cyclic peptide of 11 amino acids which binds to and inhibits the cyclophilin family of prolyl isomerases, while FK506 is a macrolide that similarly binds and inhibits a different family of prolyl isomerases, namely, the FKBPs (FK506 Binding Proteins). Despite these structural differences, both drug-receptor complexes bind to and inhibit a calcium-calmodulin–dependent protein phosphatase called "calcineurin" or PP2B (protein phosphatase 2B). This enzyme plays a central role in signal transduction from the cell surface to the nucleus during the activation of quiescent T cells[17] and is required for dephosphorylation of the cytoplasmic transcription factor NFATp; the resultant NFAT (Nuclear Factor of Activated T cells) then translocates to the nucleus. Here NFAT increases affinity of ubiquitous transcription factors (e.g., AP-1; Oct-1 and NFkB) for binding to enhancer domains of T cell-specific genes including that encoding interleukin 2 (IL2). Cyclosporin A-cyclophilin, or FK506-FKBP, inhibit the dephosphorylation of NFATp, so that NFATp remains locked in the cytoplasm where it is unable to transactivate the IL2 gene. Thus IL2 secretion is inhibited by these drugs. For tolerance induction, it is relevant to note that antigenic stimulation of T cells in the absence of IL2 tends to bias the T cell towards an anergic state which may contribute towards antigen-specific tolerance.[18,19]

There are more than 1,000 analogues of cyclosporin A, and there are quite a number of analogues of Tacrolimus (FK506). None of these has been shown to be more effective with less toxicity than the parent compounds, which still are the most effective agents in preventing IL2 production following antigenic stimulation of lymphocytes. Despite there being no apparent similarities chemically between cyclosporin and FK506, these agents have virtually identical clinical behavior with similar side effects, apart from the fact that FK506 does not increase the growth of body and facial hair.

Cyclosporin A (Sandimmune and Neoral Formulations): The original formulation of cyclosporin, "Sandimmune," was unreliable in terms of the absorption from the gastrointestinal tract. Bioavailability was interfered with by the presence of food in the stomach and the absence or presence of bile in the duodenum. A new microemulsion preparation of cyclosporin called "Neoral" is more reliable since absorption is bile-independent and use of Neoral[20,21] is now replacing Sandimmune. Once absorbed, the cyclosporin A in Sandimmune and Neoral appears to be identical in terms of its immunosuppression and side effects.[21]

FK506 (Prograf): The Prograf formulation of FK506 gives bile-independent absorption and thus provides a powerful alternative drug to cyclosporin A, especially for liver graft recipients. Importantly, FK506 inhibits the same biochemical target as cyclosporin A but is *a hundredfold more potent*. This very potency means that FK506 is effective at trough levels of 5 ng/mL whole blood (i.e., around the limits of detection) and that highly accurate monitoring is required to avoid toxic overdosing associated with trough levels equal to or greater than 20 ng/mL whole blood. In two large trials of FK506[22,23] compared with cyclosporin, there was significantly less acute and chronic rejection in patients with liver allografts treated with FK506, although there was no improvement in overall graft or patient survival.

Toxicity and Therapeutic Drug Monitoring (TDM): Unfortunately, side effects, particularly nephrotoxicity, but also neurotoxicity, hypertension, and interference with blood glucose control are the main drawbacks to cyclosporin A and FK506. Since both drugs have a low therapeutic index it is important to measure blood levels–especially in the first weeks posttransplantation when a given dose may result in wide variations in drug exposure depending on (1) absorption, (2) metabolism, (3) age, (4) type of graft, (5) type of vehicle (e.g., Sandimmune compared to Neoral), and (6) concomitant drug therapies. Desired drug levels are extremely low, being around 100 to 300 ng/mL whole blood for CsA trough level and 5 to 10 ng/mL whole blood for FK506 trough level. Specificity and sensitivity of assay procedures are very demanding, and expert biochemical backup is required to ensure safe usage of these immunosuppressants.

3. *Rapamycin*.

Rapamycin is a fermentation product of *Streptomyces hygroscopicus* from Easter Island which has anti-fungal and anti-tumor activity and is also a powerful immunosuppressant.[24-26]

Mode of Action: Rapamycin is an immunosuppressive macrolide drug chemically related to FK506 which also requires FKBP binding to form an active complex. The mode of action of rapamycin differs from that of FK506 since calcineurin is not inhibited and IL2 secretion is normal; however, in the presence of rapamycin, the receptor for IL2 is unable to complete signal transduction to the nucleus upon its activation by the secreted IL2 ligand. Thus the overall effect is to *bind* the cell to IL2 rather than prevent IL2 secretion per se. Since rapamycin and FK506 share the same intracellular receptor

(FKBP), one may compete with the effects of the other. However, cyclosporin A binds cyclophilin, a different and distinct isomerase, and rapamycin may be used to synergise with cyclosporin A without fear of antagonism due to competition for FKBP.

Rapamycin, by acting on a target required for cell cycle progression through G1, is more universally active than the FK506- and CsA-immunophilin complexes, although lymphocytes appear to be more sensitive to rapamycin than other cell types. Variation in sensitivity is likely to reflect differences in the level of the direct target of FKBP–rapamycin, combined with any central or supplementary role of these targets in signal transduction.

Clinical Potential: Since Cyclosporin A and rapamycin act to inhibit different, sequential responses during immune attack, they combine synergistically to prevent graft rejection, and combination therapies should allow reduced drug exposure. In addition, rapamycin interferes with experimental proliferative obstructive arteritis,[27] possibly by inhibiting growth factor-induced smooth muscle cell proliferation.[28] This would be valuable in preventing the intimal thickening associated with progressive chronic rejection. Rapamycin is now in phase II of clinical trials and so far does not seem to have significant nephrotoxicity or hepatotoxicity.

Other Drugs

1. Deoxyspergualin.

Spergualin is an antibiotic isolated from the bacillus Lactosporus[29] in 1981 in Japan. The synthetic spergualin analogue deoxyspergualin[30,31] prolongs allograft survival in rats. Its mode of action is not fully understood, although it inhibits lymphocyte clonal expansion in response to antigenic challenge and also interferes with antibody production.[32] It is known to bind with a heat shock protein, HSP70, and may interfere with antigen presentation and processing. It can cause leukopaenia, thrombocytopaenia, and nausea. It seems to be nontoxic to pancreatic islets of Langerhans.[33] A major disadvantage is that it has to be given parenterally.

2. Leflunomide.

Leflunomide was developed as a herbicide and is an isoxasole compound with immunomodulatory properties.[34] Its mode of action is unknown beyond a reported inhibition of tyrosine kinase activity. The tyrosine kinases perform essential cell functions, and more detail is required to identify which type or types of tyrosine kinase are inhibited by leflunomide in order to assess potential toxicity at immunosuppressive doses. Leflunomide has been used extensively in patients with rheumatoid arthritis with good results at doses which are nontoxic, but it has not been used clinically in organ grafting.

MONOCLONAL ANTIBODIES

In theory new monoclonal antibodies should give the most specific effects in immunosuppression since targeting can be directed against one epitope (their use for tolerance induction has been discussed recently by Waldmann and Cobboid[35]). There are, however, difficulties with attaining optimal results in clinical practice. Treatment with large protein molecules may not achieve access to the whole of the lymphoid and reticuloendothelial system; in addition *anti*-antibodies generated by the patient against the injected foreign immunoglobulin protein can prevent efficacy if the treatment is continued. Molecular engineering largely to replace the animal immunoglobulin–preserving only the original epitope binding site–by human immunoglobulin avoids the anti-antibody response.

Target Epitopes for Induction of Tolerance

Monoclonal antibodies (mabs) have exquisite specificity for target antigens and provide powerful reagents. Mabs against IL2, or the IL2 receptor, are directly relevant to the induction of tolerance and have been used both experimentally and clinically to reduce the IL2 signal at the time of transplantation. OKT3 is a mab directed against the T-cell receptor complex required for antigen recognition: OKT3 is immunosuppressive but may not be tolerogenic since it would prevent antigen recognition by graft-reactive T cells, a feature required for specific tolerance induction. An alternative approach which leaves antigen recognition intact is to target the CD4 co-receptor with mab. CD4 is expressed on "helper" T cells and binds to MHC class II to cooperate in signaling; in the absence of CD4, the T cell is unable to secrete IL2 in response to the presented antigen.[36] (A similar scenario applies to cytotoxic T cells where CD8 is a co-receptor reacting with MHC class I.) T cells also express CD28, which provides a separate, co-stimulatory pathway for IL2 induction and also may be targeted in therapy.[37]

Tolerance in Rodents

In vivo treatment of mice with a brief course of CD4 mab plus CD8 mab at the time of heart grafting results in stable tolerance to the graft.[38] Importantly, this tolerance is "infectious"[39,40] in the sense that potentially aggressive cells become unreactive in the presence of tolerant cells which share the same antigenic target. Thus transfer of tolerant spleen cells to a naive recipient of a donor-type graft will transfer the tolerant state. This implies that the capacity for graft-specific tolerance has been amplified, and the concept of a simple "hole" in the immune repertoire is not sufficient to explain this peripheral tolerance. Instead it is thought that graft reactive cells in the original mab-treated recipient become switched from an "aggressive" to a "suppressive" mode, and carry the suppressive regulatory property upon adoptive transfer where it is dominant over the naive recipient's graft-reactive cells.

Antiglobulin Responses

The use of mabs to interfere with the immune response to a foreign graft has been successful in rodents, but clinical application is restricted by the patient making an immune response against the foreign mab protein (i.e., an antiglobulin response). This often occurs by day 10 and effectively obviates further therapeutic effect of the mab in addition to risking side effects of an anaphylactoid nature.

Tolerance in Preclinical Models

Preclinical research is needed to identify therapeutic strategies using mabs in combination with conventional immunosuppressive drugs, and in Cambridge, England, mabs against dog lymphocytes have been generated to allow transposition of methods to achieve tolerance induction from rodents to a large animal model.[41,42] By using mabs in conjunction with azathioprine and cyclosporin A (given with four dose reductions for a total of 56 days), we have shown that CD4 and

CD8 mabs significantly prolong renal allograft survival between strongly mismatched dogs, with operational tolerance occurring in some cases. Moreover, by combining the mabs (rat immunoglobulin) with azathioprine and cyclosporin A, the antiglobulin response was prevented, opening the way to more prolonged, effective mab treatment.

Clinical Use

To date OKT3 is the only monoclonal antibody that has been licensed for clinical use. OKT3 mab therapy prevents antigen recognition and causes removal of T cells from the peripheral circulation, thereby providing a valuable antirejection therapy–especially in cases of steroid-resistant rejection. Repeated use of OKT3 is limited by the antiglobulin response. More importantly, excessive exposure to OKT3 (over 7.5 mg total) is associated with the development of early lympho-proliferative disease (ELPD) and lymphoma, probably due to loss of cytotoxic T-cell control over EBV-induced proliferation of B lymphocytes.

The use of mabs in the clinic is largely limited to induction therapies or reversal of rejection episodes, and their tolerogenic potential has yet to be realized in humans. The rodent studies have confirmed the powerful ability of mabs directed against co-receptor, co-signal, or adhesion molecules to induce donor-specific tolerance to grafts. Extrapolation to the clinic awaits guidance from preclinical models where therapeutic strategies for reliable tolerance induction in large mammals may be identified. High cost, availability of species-specific mabs, and protracted readout times are likely to underlie the slow progress to date.

CONCLUSION

For the induction of donor organ-specific tolerance, the immune system needs to engage graft antigens (to provide specificity) coincident with therapy which alters immune regulation away from aggressive and towards suppressive responses. Antigen engagement in the absence of IL2 favors suppressive responses, so reagents which interfere with IL2 secretion (cyclosporin, FK506), IL2-mediated signaling (rapamycin), or monoclonal antibodies, which block co-receptor help for IL2 secretion, will each contribute to tolerance induction. In large animals and in humans, the large mass of the lymphoreticular system is difficult to control fully during allogeneic responses against the graft, and here there is value in reductive therapy using drugs or depleting anti-lymphocyte antibodies, plus antimetabolites to control early amplification responses during the critical period required to induce tolerance. Evidence for depletion synergising with tolerogenic protocols is available in mouse and preclinical models. Similarly, reduction of antigen presentation (cyclosporin, deoxyspergualin) may reduce aggressive responses and so alter the threshhold in favor of suppressive immune regulation. The finding that combined cyclosporin and azathioprine inhibit the antiglobulin response opens up the powerful potential of repeated mab therapies, although this does not extend to OKT3 which may be reserved for steroid-resistant rejection responses to avoid OKT3-related ELPD.

By combining up-to-date clinical and scientific information, we should soon identify how best to use these new reagents in the context of the established agents cortisone, azathioprine, and cyclosporin. It is important to remember that excessive immunosuppression is not our major goal. It may be necessary to spare certain intrinsic immune activity, or even to augment certain regulatory actions, in order to establish tolerance. Indeed, it has been hypothesized[43] that a window of opportunity for immunological engagement (WOOFIE) between donor and recipient is an integral component of tolerance induction, both providing specificity for donor-type antigen and initiating a means to retain donor-type antigen in a form suitable for the maintenance of donor-specific tolerance. STI

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