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Immunosuppression

By CHARLES B. CARPENTER, M.D.

Deliberate attempts to interfere with a patient's immune system were not realistically considered until the transplantation era began in the 1950s. Once the surgical techniques for transplanting a kidney to a site inside the body were perfected – as proven when donors were identical twins of the recipients[1]– the need for an effective means to prevent immunologically-mediated rejection of the graft became top priority. The first protocols employed whole body irradiation in sublethal doses, followed by doses of hydrocortisone.[2] This was shown to be effective in centers in Boston and Paris, but only in non-identical twins who were later noted to share all HLA antigens.

The development of orally administered chemical immunosuppression came shortly after experiments in immunized rabbits[3] that demonstrated the immunosuppressive qualities of 6-mercaptopurine (6-MP), the first effective drug for treatment of acute lymphoblastic leukemia. A small number of patients receiving renal transplants with 6-MP had some beneficial effects, but clinical enthusiasm was low until it was shown in a series of dog kidney transplant experiments that one of the modified 6-MP molecules, azathioprine, was effective. Although only a fraction of the azathioprine-treated dog transplants survived beyond a few months, initial clinical application began in 1962.[4] It was only after azathioprine was combined with steroids that it was it shown to produce 40%-50% graft survivals at 1 year.[5] Not all patients died from uremia, since the high steroid doses promoted fatal infections. It was feared that reducing steroid exposure would promote premature graft loss which, in the days prior to the development of chronic dialysis systems, was a death sentence. With the later availability of access to chronic dialysis, the 1-year survival rates progressively improved to 60%-70 % by 1983, when cyclosporine was approved and started to make an impact.[6]

Approaches to immunosuppression

There are two avenues that can be used to prevent the activation of cells of the immune system. One is to alter various pathways *inside* the cell by chemical inhibitors that diffuse or are transported through cell membranes. The other is to provide inhibitors that engage specifically targeted molecules *on* the cell surface. The former includes products of nature or are synthetic, and are processed or manufactured as classical chemical drugs of a relatively small size that can be administered by mouth. The latter are larger proteinaceous molecules that are injected into a patient's veins. These can be antibodies that cause the disappearance of mononuclear leukocytes from the blood or they may be specific antibodies to surface molecules, thereby blocking or removing selected functional molecules. Increasingly, constructs of antibody-like agents that consist of a natural ligand or receptor linked to the Fc portion of human IgG are being tested.

There are general criteria for the development of safe and effective immunosuppressive agents of both the chemical and biological types (Table 1). These criteria are most applicable to patients who are not already immune to histocompatibilty antigens, and to HLA, in particular. Patients immunized to histocompatibility antigens frequently require higher doses of the more powerful agents and run a greater risk of infections. The availability of appropriate antibiotics targeting DNA viruses has made it possible for such patients to survive a higher degree of nonspecific immunosuppression.

Chemical immunosuppression

Azathioprine: This agent is a purine analog that can inhibit synthesis of DNA, RNA, or both.[7] The primary immune response requires a complex series of cell-to-cell interactions that lead to proliferation and expansion of lymphocytes. This drug is effective for inhibiting mitosis of immunologically-competent lymphoid cells. Azathioprine has little effect in suppressing a secondary immune response and is ineffective in sensitized patients or for treatment of acute rejection episodes. The drug also affects the bone marrow with variable reductions in circulating mononuclear cells, both lymphocytes and macrophages, and also granulocytes. Leukopenia is the principal side effect that limits the amount that can administered. Anemia also occurs, but is rarely severe, and thrombocytopenia is less common.

Table 1: Ideal criteria for immunosuppressive agents

- Major effect is directed to primary, and not secondary immune responses, to avoid affecting memory cells for infectious agents
- Site of action limited to the immune response, with minimal effects on other tissues
- Amenable to combinations with other agents for synergy
- Sparing of pathways that lead to development of regulatory cells

Azathioprine is usually combined with prednisone and cyclosporine for maintenance therapy, using 1.5 to 2.0 mg/ kg per day orally. It is rapidly metabolized by the liver and does not routinely require dose reductions in the presence of renal failure. Dosing is guided by the white blood cell count. Since more effective drugs are currently in use, azathioprine is almost never used in new transplants; however, long-term patients with successful transplants may still be taking this drug. The interaction between azathioprine and allopurinol is important as the latter is an inhibitor of xanthine oxidase, which catabolizes azathioprine.[8,9] Patients who receive allopurinol for gout must have their azathioprine dose reduced to avoid severe leukopenia that develops because of the longer half-life of azathioprine. A wiser approach is conversion from azathioprine to mycophenolate mofetil.

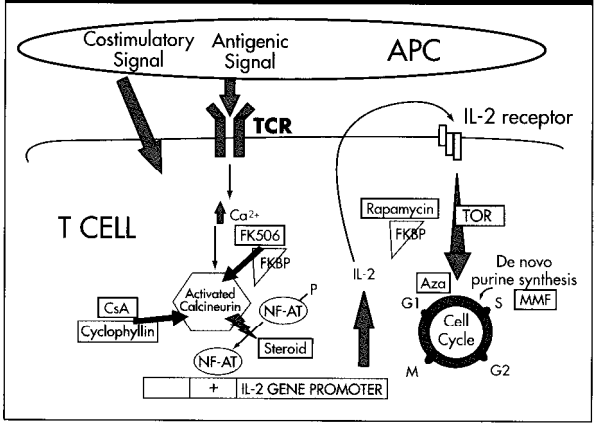
***Corticosteroids*:** By the time azathioprine was in use, glucocorticoids (eg, prednisone and prednisolone) were available and known to have powerful effects on inflammation. Steroids of this class greatly improved the ability of azathioprine to prevent rejection and, when acute rejection did develop, large pulse doses of glucocorticoids (ie, 1000 mg intravenously) were frequently capable of reversing acute rejection readily, though not in all cases.

The current understanding is that steroid effects are mostly on the macrophage and dendritic antigen-presenting cells, with the key effects being inhibition of IL-6 and IL-1 gene expression in these cells.[10,11] As part of the innate immune system, these cytokines are generally activated by antigen non-specific stimuli, such as ischemic hypoxic injury. It is now known that the innate system is a major accelerator of adaptive antigen-specific immunity. As concentrations of glucocorticoids rise in the blood, they enter the cytosol, bind to a specific receptor, and this complex migrates to the nucleus to bind regulatory regions of DNA. This results in diminished activation of several genes, such as IL-2 (Figure 1).

In addition to a broad generalized inhibitory effect on all immune responses, there are many other well-known complications associated with high-dose or prolonged steroid use. When steroids are rapidly tapered, patients are at risk for a "breakthrough" rejection.[12] Even a slow taper to low doses or discontinuation in stable patients may later result in deterioration of renal function.[13,14] However, at present, a change to newer, more effective drugs (discussed below) can reduce the dangers of steroid withdrawal.

***Mycophenolate mofetil (MMF)*:** This chemical compound is another inhibitor of nucleotide metabolism[10,15] and has replaced azathioprine for new patients. In comparison with azathioprine, it has increased immunosuppressive effects with less bone marrow suppression. When ingested, MMF is converted to mycophenolic acid (MPA), a reversible inhibitor of

Figure 1: Schema of T cell activation pathways and sites of inhibition of chemical immunosuppressant agents



The T Cell Receptor (TCR) complex provides *signal 1*, when a specific antigen is presented by the HLA molecules of the antigen-presenting cell (APC). Co-stimulatory signals (*signal 2*) from other APC interactions with the T cell assure a robust and stable response via a calcium-dependent pathway that results in the activation of calcineurin, which is then able to dephosphorlyate NF-AT that then can move from the cytoplasm to the nucleus where it binds to the promoters of several cytokines, as illustrated by IL-2. *Signal 3*, originating with the engagement of cytokine receptors, activates the cell cycle for DNA replication and cell division, via the target of rapamycin (TOR) to the phosphorylation of p70 S6 kinase. Cyclosporine (CsA) and tacrolimus (FK506) prevent the activation of calcineurin, but not in the same manner. Cyclosporine binds to a particular small protein, cyclophilin, and tacrolimus binds to FK binding protein (FKBP), but each of these new heterodimers then binds to calcineurin , blocking activation of that molecule, and thereby preventing production of IL-2 and other cytokines. Corticosteroids also interfere with transcription of cytokine genes. While sirolimus also binds to FKBP, the sirolimus heterodimer binds to TOR, thereby preventing the cell from responding to the cytokine signals for proliferation. Azathioprine and mycophenolate mofetil block *de novo* purine synthesis after the cell cycle is initiated. Clinical trials of various combinations of agents are continuing, and there is an indication of improving clinical results using moderate doses active in different pathways.

inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme that promotes *de novo* synthesis of guanosine nucleotides and nucleosides. In the lymphocytes, there is a selective block in both T and B cell proliferation, in contrast with other tissue cells that have an alternative salvage pathway capable of bypassing the blockade by MPA.

MMF can also replace azathioprine in patients who need allopurinol therapy for gout. In the first 3 clinical trials of MMF (at a dose of 2 grams/day), there was an average 50% reduction in the incidence of acute rejections in comparison with placebo or azathioprine. In conjunction with steroids and cyclosporine, the absolute incidence dropped from 40% to 20%.[16] In follow-up, some improvement in long-term survival rates has also been noted.[17] MMF was approved for clinical use in 1995.

Results in African Americans demonstrate that a higher dose (3 grams/day) is required for any benefit.[18] Side effects are mostly gastrointestinal irritative symptoms that usually respond to dose reduction and, when leukopenia develops in the setting of other potential marrow depressants, dose reduction in MMF may be helpful. Blood level monitoring of the active moiety, MPA, has revealed variable pharmacokinetics from patient to patient, but there is no essential need for blood level monitoring in routine cases.

***Cyclosporine*:** This drug is a small hydrophobic cyclic peptide derived from a Norwegian fungus that was one of many

natural molecules being evaluated for antifungal antibiotic activity in the 1970s. Although cyclosporine had some antifungal activity, it was taken off the list when a toxic screen in mice indicated that it had immunosuppressive qualities. It was shown to prevent acute rejection activity in animals and subsequently, limited clinical trials began. With cyclosporine, dose-dependent nephrotoxicity is caused by the native molecule. As with azathioprine, its benefits are limited to prevention of rejection and it is not effective in reversing rejection activity, unless the rejection is the result of inadequate dosing. Cyclosporine was approved for clinical use in 1983 and, by 1985, the number of deceased donor kidney transplants at one year was clearly improving. Although some centers used cyclosporine in place of azathioprine, a consensus emerged that triple therapy with steroids permitted easier management, allowing some lowering of doses for each agent.

Because of advances in understanding the roles of IL-2 and other cytokines in immunity, it was possible to pursue fruitful mechanistic studies on the mode of action of cyclosporine in the 1980s (Figure 1). In vitro, it was shown that the addition of cyclosporine to antigen-stimulated cell cultures prevented secretion of IL-2, IL-3, IL-4, IFN-γ, and TNF-α from T lymphocytes, while macrophages could still make TGF-ß and IL-1.[19,20] Suppressed proliferation of T cells in vitro could be restored by the addition of IL-2. The intracellular pathway most affected was the calcium-dependent pathway to NFκB. In vitro, cyclosporine bound tightly to a family of cytosolic molecules, named the "cyclophilins." One of these is most abundant in lymphocytes. The cyclosporine-cyclophilin complex can then bind to a third molecule, calcineurin, thereby blocking the latter's activity as a serine threonine phosphatase.[21,22] Calcineurin normally dephosphorylates cytoplasmic NF-AT (nuclear factor of activated T cells), permitting it to be transported to the nucleus where it initiates transcription of genes such as *Fos* and *Jun*. When blocked, synthesis of IL-2 and the other cytokines mentioned are greatly reduced and proliferation is inhibited.

Clinical results from tens of thousands of renal transplants have been impressive. Cyclosporine is usually administered orally in doses of 2-4 mg/kg bid, and monitored by blood levels, usually obtained just before a dose. The experiences in the early 1980s included difficulties with dosing in relation to renal function, differentiating nephrotoxicity from rejection, confronting variabilities in absorption, a lack of standardized blood level testing, and poor understanding of the effects of other drugs on cyclosporine metabolism. All of these difficulties have been mitigated with experience, including better laboratory assays and the development of a microemulsion to improve absorption. However, the nephrotoxicty problem remains, as well as hirsutism, tremor, gingival hyperplasia, and some increased risk of hepatotoxicity and diabetes. The latter is less likely when steroid doses are low.

Tacrolimus: The successes with cyclosporine led to the investigation of other fungal sources as immunosuppressive drugs. In 1994, another calcineurin inhibitor, tacrolimus (FK506) from Japan, was approved for clinical use. The interesting thing about this molecule is that it is a macrolide, a completely different structure from the peptide cyclosporine. It has a totally different binding protein (FKBP12) from the cyclophilins for cyclosporine, yet the complex of tacrolimus with FKBP12 also binds to, and blocks, the effects of calcineurin.[23] An expanded term, immunophilin, was adopted to include both the cyclophilins and FKBPs. The effect on calcineurin is essentially the same as cyclosporine. Tacrolimus has a similar half-life to that of cyclosporine, but its effective and relatively non-toxic doses by weight are much lower, in the range of 0.05-0.10 mg/kg bid. More important is the proven increased potency of tacrolimus in preventing rejections[24] and in reversing rejections in patients taking cyclosporine. Tacrolimus does not produce hirsutism and gum hyperplasia, and it has a lower tendency to raise blood lipid levels or blood pressure. It can be a potent neurotoxin, nephrotoxin, an inducer of diabetes, and a potentiator of MMF gastrointestinal side effects; however, these data come from trials in which higher doses were in use. The proportion of new patients started on tacrolimus has risen sharply in the last 2 years.

Sirolimus (Rapamycin): Another fungal product was discovered during the screening of fungi from Easter Island (Rapanui); it was initially named rapamycin. It was also unsuitable for use as an antibiotic because of its effects on the immune system, so it sat on the shelf for several years before undergoing further evaluation. When compared with the two calcineurin inhibitors, sirolimus has no effect on the calcineurin pathway since generation of IL-2 and other cytokines is normal, while T cells are made unresponsive to IL-2.[25] The macrolide structure of sirolimus is similar to that of tacrolimus and they have the same binding site for FKBP12. In the next step, sirolimus-FKBP12 binds to a previously unknown protein named mTOR (mammalian target of rapamycin), and not to calcineurin.[23] The resulting complex, consisting of sirolimus-FKBP12-mTOR, then binds and inhibits phosphorylation of p70 S6 kinase, thereby blocking cell cycle activity that is promoted by co-stimulatory and cytokine signals (Figure 1). Because of its effects on cell proliferation, sirolimus also slows proliferation of B lymphocytes and fibroblasts.

Clinical trials[26] have established that sirolimus has a relatively long half-life after oral administration, requiring only once-a-day dosing, except in children who may need twice daily doses. Monitoring blood levels can be useful in establishing doses that produce effective therapy without side effects. Adverse effects include delayed wound healing, mouth ulcers, hyperlipidemia, anemia, and thrombocytopenia. Some studies have studied sirolimus in the absence of calcineurin inhibitors,[27] while others have examined its combination with other agents or with deletion of steroids.[28,29] Since sirolimus also inhibits proliferation of fibroblasts, its potential for preventing the development of chronic allograft nephropathy is awaiting the results of trials. It has already been demonstrated that sirolimus prevents the failure of coronary artery stents when they are coated with the drug.

Interactions of immunosuppressive drugs with other medications

There are two categories of potentially dangerous drug combinations in immunosuppressed patients.

The first category is bone marrow suppression, which most commonly occurs with azathioprine. It also occurs with mycophenolate, especially if there is concurrent administration of other marrow suppressants, such as the antiviral drugs. Leukopenia may also occur during a viral illness, especially

with cytomegalovirus. In these cases, clinical judgement is needed to decide whether the immunosuppressive or the antibiotic drug should be reduced.

The second category involves all 3 of the fungus-derived drugs – cyclosporine, tacrolimus, and sirolimus – as they are catabolized by the same hepatic cytochrome p450 systems (CYP3A4/5) that are involved in the degradation of some other commonly used drugs.[31] Prior to reaching the liver, much of the orally administered drug is either degraded or prevented from being absorbed. CYP3A4/5 is also present in the intestinal wall[32] and oral absorption is reduced by the multi-drug resistance gene, *MDR-1*. This gene produces a transmembrane protein, P-glycoprotein (P-gp)[33] that actively transports a large number of molecules (including cyclosporine, tacrolimus, and sirolimus) back into the intestinal lumen.

The activity of both of these systems varies greatly from individual to individual and there may be both genetic and environmental factors at play. Stable patients on maintenance doses generally remain well for long periods on dosing that was established in the first months, unless they are given a new drug that alters the metabolism of the maintenance drugs, and a large rise or fall in blood level of the drug develops. There are drugs that inhibit CYP3A4/5 activity and others that stimulate it. Depending on the status of the patient, a rise in the blood level could produce a significant side effect, while a fall in blood level could provoke a rejection episode.

Table 2 lists some of the more common drugs that are particularly prone to produce changes in blood levels. In any immunosuppressed patient who has developed impaired renal function or drug side effects, it is important to ascertain if there have been any changes in medications, including the herbal variety. Pharmacists now have databases that can alert them to these incompatibilities.

Because of the competition for CYP3A4/5, combinations of two immunosuppressive drugs can change the metabolism of both drugs. Clinical trials to test whether combinations can improve therapeutic effects, while reducing side effects, have provided some information, not all of which was predictable. For example, if sirolimus is used with tacrolimus, tacrolimus levels are lowered,[34] but if it is used with cyclosporine, cyclosporine levels increase.[35] Further, if MMF is used with tacrolimus, MPA levels go up,[36] however, with cyclosporine, MPA levels lower.[37] Considering the known complexity of catabolic systems and variations between patients, it appears that only empiric observation in ongoing clinical trails will establish the optimal protocols for combinations of chemical immunosuppressants.

Table 2: Common drugs that alter blood levels of cyclosporine, tacrolimus, and sirolimus

Increased levels (inhibit catabolism)

- diltiazem, nicardipine, verapamil
- clarithromycin, erythromycin
- fluconazole, itraconazole, ketoconazole, voriconazole
- danazol
- chloroquine
- estradiol
- amiodarone
- grapefruit juice

Decreased levels (accelerate catabolism)

- phenobarbital
- carbamazepine
- dexamethazone
- phenytoin
- rifampin
- St. John's Wort

Antibodies and genetically-engineered protein inhibitors

Polyclonal antibodies: During the two decades when the main immunosuppressive therapy consisted of azathioprine and steroids, there was a lot of activity in creating antibodies (mostly in horses or rabbits) against human lymphocytes (eg, cultured B cell lines or thymocytes). The gamma globulin fraction of animal serum was initially injected into muscle and later, with better purification, by the intravenous route. It was first established that the reversal of acute rejection was more reliably accomplished with antilymphocyte globulins (ALG) than with steroid pulses.[38] Clearance of leukocytes from the blood is rapid after administration and lymphopenia can be profound, with gradual reconstitution thereafter. A serum sickness syndrome may develop in patients who become sensitized to the foreign proteins. The use of ALG or antithymocyte globulin (ATG) was next extended to so-called "induction" therapy, either to cover the early omission of cyclosporine, thereby sparing nephrotoxic effects, or hypothetically as a means to induce better "tolerance." A rabbit ATG, prepared from large pools of animal donors immunized with portions of human thymuses removed during cardiothoracic surgery, is the product most used today.[39] The lymphopenia obtained with this product is more prolonged than with other polyclonals. Such mixtures of IgG antibodies contained a large number of antibodies that are not reactive with lymphocytes. When the timings of intraoperative vs. postoperative administration of rabbit ATG were recently compared, there was less delayed graft function and acute rejection in the intraoperative group.[40] This suggests that some antibodies in the ATG may ameliorate the effects of initial ischemic injury if given at the time of surgery.

Monoclonal antibodies: The development of the technology to make monoclonal IgG antibodies[10] that have specificity to single antigenic sites has become commonplace. The first application to clinical transplantation was with anti-CD3 (OKT3).[41] The concept of blocking the function of a limited number of recipient cells was put to the test as anti-CD3 recognized a site on the ε chain of the T cell receptor (TCR). It was first shown to reverse a very high percentage of acute rejections, including those resistant to steroid pulses and subsequently, was used for induction as well.[42]

In vivo, OKT3 produces a variable, but sometimes severe reaction, with the first and second administrations. The reaction consists of chills, fever, GI upsets, headache, and pulmonary edema in over-hydrated

patients, as well as a delayed onset of aseptic meningitis or serum sickness. It was soon demonstrated that these side effects were the result of a massive release of cytokines, with TNFα being most important. In vitro, it was demonstrated that OKT3 was a mitogen for T cells.

When OKT3 is compared with polyclonal ATG, the effectiveness is equivalent, while the polyclonals are better tolerated.[43] Another consequence of OKT3 is the risk of opportunistic infections and post-transplant lymphoproliferative disease (PTLD), especially after repeat courses of OKT3.

After murine monoclonal antibodies against the IL-2 receptor α chain were shown to prolong kidney allograft survival when used alone in monkeys,[44] limited clinical trials were undertaken in the late 1980s in conjunction with standard triple drug therapy (cyclosporine, azathioprine, and steroids).[45,46] Onset of acute rejections was delayed for several days until antibodies to the murine protein developed, causing loss of activity.

Further development was delayed until humanized versions of antibodies were available in the 1990s. This was accomplished by taking portions of genes from mice that encode the combining sites only (daclizumab),[47] or a larger region of the light chains and distal portion of the murine heavy chains (basiliximab),[48] and splicing them with human genes for the rest of the IgG molecule, including the entire Fc portion. This breakthrough provided two important benefits: a marked reduction in the immunogenicity of the foreign protein and a more physiological survival time in the vascular space. These antibodies engage the high-affinity IL-2R α chain (also known as CD25) and internalize it, leaving intact cells with only the low-affinity β chain, along with the signaling γ chain.[49] There are virtually no adverse reactions with such humanized antibodies[50] and, in most patients, they improve early graft survival rates.

There are several other humanized monoclonal antibodies being studied in trials or under development. Active now is Campath 1H(anti-CD52), a shared molecule on perpiheral leukocytes that produces profound leukopenia for several weeks, and other constructs, such as CTLA4Ig and similar molecules related to other costimulation pathways of T cell activation. Humanized monoclonal antibodies against several cytokines are also in trials of autoimmune diseases, and toxins can be attached to monoclonal antibodies in order to delete targeted cells.

Conclusions

There are differences of opinion regarding the optimal goals of future research of immunosuppressive regimens. Careful clinical study of combinations of agents, both chemical and biological, with special attention to dosing and timing, may result in the elimination of serious side effects, while preventing all rejection activity. Alternatively, protocols designed to induce a form of tolerance that is free from maintenance requirements may be possible. One cannot assume that these approaches can be easily mixed since the use of chemical agents can sometimes prevent the development of tolerance in animal models. Also, in either case, one cannot ignore the powerful differences between naïve and presensitized recipients with respect to the quality of clinical results. In particular, it may be much more difficult to achieve a tolerant state in immunized patients. There is some evidence that gene polymorphisms of immune response molecules, both innate and antigen-specific, are associated with different propensities for tissue injury. If confirmed, such genotypes may help in tailoring optimal protocols to the individual patient.

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Upcoming Scientific Meetings

28 August – 1 September 2004
First Joint Congress of the International Society for Peritoneal Dialysis and the European Peritoneal Dialysis Meeting
Amsterdam, The Netherlands
CONTACT: Jan Van Goyenkade
Tel: 11 +31.20.673.7306
Email: spd-europe@eurocongress.com
Website: www.ispd-europd2004.org

5-10 September 2004
XX International Congress of the Transplantation Society
Vienna, Austria
CONTACT: Email: transplantation2004@mondial.at
Website: www.transplantation2004.at

1-2 October 2004
3rd Annual Conference on Prevention in Renal Disease
Toronto, Canada
CONTACT: Dr. D.G. Oreopoulos
Tel. +1 416.603.7974
Email: Sandra.Medeiros@uhn.on.ca.
Website: www.nephroprevention.com

27 October - 1 November 2004
Annual Meeting & Scientific Exposition of the American Society of Nephrology (Renal Week)
St. Louis, Missouri
CONTACT: ASN 202-367-1190
Email: asn@dc.sba.com
Website: www.asn-online.org

8-11 December 2004
The 3rd International Congress on Immunosuppression
San Diego, California
CONTACT: Renee.Bartley@pwnj.thomson.com.

Dr. Carpenter has reported no relationships with industry relevant to the enclosed CME program.