CALCINEURIN INHIBITOR SPARING PROTOCOL IN LIVING DONOR PEDIATRIC KIDNEY TRANSPLANTATION
(CN01)

AMENDMENT 2: September 13, 2002

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Study Activated
February 13, 2001

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SCHEMA

Improvements in post transplant care and immunosuppression have led to increasingly successful short-term outcome in pediatric recipients of kidney transplants. Unfortunately, morbidity associated with immunosuppressive agents is substantial and some of these drugs may accelerate chronic allograft nephropathy. We propose that the use of the TOR-inhibitor, sirolimus, will obviate the need for calcineurin inhibitors in post-transplant immunosuppression in recipients of living donor grafts. If this pilot study is successful, we will extend the protocol to include cadaver donor recipients and to a protocol including pre-transplant donor specific transfusions (DST) under sirolimus coverage. This deliberate pre-transplant exposure to donor antigen may lead to donor-specific hyperesponsiveness and to the opportunity to reduce chronic immunosuppression even further. This long-term proposal is based on preclinical observations that DST plus T-cell costimulatory blockade (rapamycin) may result in activation induced cell death (AICD) of alloreactive T cells that are harmful to the graft. These protocols will include intense immunologic monitoring which is designed to uncover anti-donor responsiveness as early as possible.

The living donor kidney transplant entering this study will receive the following immunosuppression:

Inductive Immunosuppression
    Patients will receive humanized anti-CD25 monoclonal antibody DACLUZIMAB, administered in 5 doses over a 2 month period. The first dose will be administered intra-operatively and the subsequent doses will be administered every two weeks up to 8 weeks post transplantation.

Maintenance Immunosuppression
1.   Sirolimus (Rapamune):

    Administered daily beginning on Day -1. The first dose will be $10mg/m^2$ and subsequent doses will be 3 mg/m^2 2 times a day as tablets or liquid. The dose will be adjusted to maintain the 24-hour trough level at 20-25 ng/mL for the first two months and at 20 ng/mL for the next four months and 15 ng/mL thereafter. Blood will be obtained for daily levels until stable levels have been maintained. Thereafter, for the first two months, levels will be obtained at least twice weekly. Thereafter, levels will be obtained at each outpatient visit. Also, 24-hour sirolimus pharmacokinetic studies will be determined at months one and three in each patient after stable levels have been obtained.

2.   Mycophenolate Mofetil:

    Administered daily beginning on Post-Op Day 1. The dose will be 1200 mg/m^2/day administered as a pill or liquid 2-3 times per day.

3.   Corticosteroids:

    (A) Methylprednisolone will be administered at 10 mg/kg (maximum 700 mg) intravenously, peri-operatively and on Post-Op Day 1.

    (B) Prednisone will be administered orally beginning on

Post-Op Day 2. The dose will be 2 mg/kg/day, divided BID, maximum dose 60 mg. Starting on Day 4 the dose will be reduced to reach 0.5 mg/kg/day by Day 19 and then maintained, unchanged, until Post-Op Day 30. Starting on Day 30 prednisone dose will be reduced every two weeks by 0.125 mg/kg to reach 0.25 mg/kg/day, QD, by Post-Op Day 60. On Post-Op Day 60 the dose will be further reduced by 0.05 mg/kg to achieve a dose of 0.2 mg/kg/day. This dose will be maintained through Post-Op Day 180. On Post-Op Day 180, taper prednisone to 0.15 mg/kg/day administered on alternate days at the discretion of the investigator (max 15 mg q.o.d.)

1.0 BACKGROUND AND RATIONALE

Renal transplantation is widely recognized is the treatment of choice for children with End Stage Renal Disease (ESRD) [1]. A functioning renal transplant enables children to develop normally, grow reasonably well and normalize their school performance levels[2]. Thus, pediatric programs attempt to transplant children with ESRD as expeditiously as possible.

There have been very few prospective, randomized, controlled trials of immunosuppression for children following renal transplantation. Virtually all drugs used have been tested only in adults and their use has been extrapolated to children. It took several years for pediatric cyclosporine dosing to be completed[3-6]. Pediatric pharmacokinetic studies of Neoral were performed soon after its release, but controlled clinical trials in pediatric renal transplant recipients have only recently been performed even though some uncontrolled reports had suggested complications of its use in children[7]. Those studies are not yet complete. Tacrolimus has been used only in an uncontrolled fashion in pediatric renal transplantation[8-10]. The pharmacokinetics of mycophenolate mofetil (MMF) in children were studied prospectively and pediatric specific doses were established soon after its general release[11]. Also, the interleukin-2 receptor monoclonal antibodies (mAbs) were studied in pediatric patients about the time of their FDA approval[12]. But neither MMF nor these antibodies has been studied in rigorous or controlled fashion in children. Retrospective data from the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS) has shown a beneficial effect of antibody induction in children, particularly infants[13]. Surprisingly, a recently completed controlled trial of OKT-3 antibody induction in children has shown no beneficial effect of this induction protocol and perhaps a detrimental effect in recipients of living donor transplantation[14].

It is not surprising, therefore, that there is no consensus about the optimal immunosuppression for children. Cumulative data from NAPRTCS suggested that slightly more than one-half of pediatric renal transplant recipients have received antibody therapy (40% ALG/ATG, 16% OKT-3) in the first month post-transplant[15]. About 80% of all pediatric transplant recipients receive triple immunosuppression (prednisone, azathioprine/MMF, cyclosporine/tacrolimus) [16]. In 1996 tacrolimus was used in 2.5% of pediatric renal transplants at day 30; in 1997 it had increased to 10.8%, but continued expansion appears limited by concern about its side effects. Several studies have suggested that the use of alternate-day steroids is safe and has substantial benefits for children[17, 18], but only 16% of pediatric renal transplant recipients are receiving steroids on an alternate day schedule one year post-transplant[16].

Despite the lack of consensus, short term outcomes of kidney transplants for children have improved during the past decade, as they have for adults[16, 19]. The NAPRTCS 1998 Annual Report shows that the one-year cadaver donor graft survival has improved from 71.7% in 1987 to 88.4% in 1996The living donor one-year graft survival has improved from 88.1% to 93% during the same time period[16]. The reason for this improved outcome has not been identified and is likely to be multi-factorial. Importantly, there is general consensus that immunosuppression protocols generally have been improved, but this may have occurred at the cost of increased side effects.

In a review of over 5000 transplants in a 10-year NAPRTCS study 49% of live donor and 63% of cadaver patients have had one or more rejection episodes by the end of the first year post-transplant[20]. NAPRTCS noted that when reviewed by age groupings, rejection ratios, time to first rejection and the recipients less than six years of age had significantly increased irreversible rejections leading to graft loss[21]. There are conflicting data about whether infants and small children have a "heightened" immune response. Some indirect evidence suggests a more vigorous immune response especially in infants. Data from the UNOS registry show a higher rate of acute rejections in young children after both living donor and cadaver donor transplantation, and adolescents were noted to have a higher rate of late acute rejections[22]. However, data from a large pediatric transplant program demonstrated that infants have a lower rate of acute rejection than older children[23]. UNOS data demonstrate that except for the youngest recipients (less than 2 years of age), 1-year pediatric renal transplant survival rates are comparable to those in adults[22]. Interestingly, data from UNOS[22] and from NAPRTCS (D. Stablein, personal communication) demonstrate that for recipients with retained graft function at one year, the youngest recipients had the longest estimated graft half-lives. Data from the most recent UNOS Annual Report [24]demonstrate substantially better 5-year patient survival in children than in older adults. Children 6-10 years of age have a 97.4% 5-year patient survival following live donor transplants, compared to 90.3% for 35-49 year olds and 81.9% for 50-64 year olds. Therefore, although it is still controversial whether children have an early heightened immune response to allografts, there is no definite evidence to suggest that it would be more difficult to induce tolerance in young recipients and perhaps there are indications that it may be easier.

A major distinguishing feature of pediatric transplant recipients is the need for children to grow. Growth failure often begins early in the course of chronic renal insufficiency. It has been suggested that a functioning transplant would enable the child to achieve catch-up growth[25]. Unfortunately, catch-up growth has been seen in only 47% of the children between the ages of two and five years, and for children over the age of five years, little catch-up growth has been noted[26]. Individual center studies have adopted a variety of techniques, such as discontinuation of prednisone[27], alternate day steroid therapy[17, 18, 28] or the use of recombinant human growth hormone[29]; however, the best catch-up growth is seen in patients who are completely withdrawn from steroids[9, 30]. Numerous uncontrolled studies have shown that steroids often can be withdrawn from children post-transplantation[9, 31]; however, acute rejection tends to occur shortly afterwards in many of these patients[32], with marked detrimental long-term effects. There is not yet a reliable immune marker that can identify patients who are hyporesponsive and/or patients who can safely undergo steroid withdrawal[30]. Post transplant growth us also inhibited by renal dysfunction, similar to what is well described in chronic renal insufficiency. As GFR declines post transplantation, growth rates inexorably fall off. Thus, protocols are designed to permit maximum growth post-transplantation must strive to maximize renal function.

Infection is generally the major cause of death of children, particularly in the first post-transplant years[13]. Other major causes include cancer/malignancy, cardiopulmonary causes and dialysis related complications[33]. Mortality after 10 years post-transplant seems to be related primarily to cardiovascular causes[34], which may be linked to the hyperlipidemia and hypertension associated with chronic immunosuppression. Indeed, chronic immunosuppression results in multiple short- and long-term complications in

children. Infections account for the majority of complications post-transplantation in children and are the principle cause of early mortality. Prophylactic therapy against the more common infections is employed by most centers at substantial costs which has its own complications. Among these are CMV infection[35-37], Pneumocystis Carinii[38], and varicella[39-42]. Post-transplant lymphoproliferative Disease (PTLD) had been reported as a complication of pediatric organ transplantation for many years[43] but the number of published reports seems to be increasing[44, 45] which may be the unfortunate consequence of the "improved" immunosuppression noted above[46]. A recent analysis has shown that young age, donor source or use of anti-lymphocyte antibodies are not risk factors for PTLD; but the use of tacrolimus appears to be associated with a 10-fold increase in incidence of PTLD[8, 45-47]. Other types of neoplasia have also been reported in pediatric renal transplant recipients[48].

Immunosuppressive therapy is also associated with significant side effects, some of which are particularly troubling to children. Cyclosporine causes substantial nephrotoxicity[49], hepatotoxicity[50], and cosmetic issues including hypertrichosis, facial dysmorphism[51] and gingival hyperplasia[52]. Tacrolimus is associated with the same degree of nephrotoxicity, but may have less cosmetic side effects[10]; however, neurologic complications, diabetes and PTLD seem to be more common[53-55]. Both Cyclosporine and Tacrolimus cause an immediate decrease in GFR which can cause metabolic and pharmacokinetic problems[49]. More importantly, these medications may have a chronic effect on kidneys that leads to a progressive loss of renal function. GFR typically declines over time in all renal transplant recipients[16] and this phenomenon, which is characterized pathologically by interstitial nephritis and intimal hyperplasia is generally attributed to "chronic rejection". However, about a substantial number of cyclosporine treated pediatric heart transplant recipients have ESRD[56]. This situation is generally attributed to the nephrotoxicity of cyclosporine and the pathologic findings in their kidneys is very similar to that described from chronic rejection. The incidence of hypertension post-transplant is demonstrated in a NAPRTCS study to be 59% at 24 months[57]. The two most widely used immunosuppressives, cyclosporine and prednisone, both exacerbate pre-existing hypertension. Both steroids and cyclosporine induce hyperlipidemia[58-60]. MMF causes leukopenia and gastrointestinal side effects. Corticosteroids causes growth retardation, aseptic necrosis, cushingoid faces and acne. Finally, although both cyclosporine and tacrolimus have improved short term graft survival rates, experimental studies have suggested that the blockade of apoptosis associated with their use may, in fact, hinder tolerance induction, thus limiting their long-term usefulness.

Thus it is clear that renal transplantation can provide substantial benefit and rehabilitation for children with ESRD. And, outcomes of renal transplantation in children have improved during the past decade. This success, however, has been limited by the incomplete and temporary nature of the non-specific tolerance produced by current long-term immunosuppressants and by the frequently serious complications associated with their use. Thus, future protocols are designed to achieve current outcomes with more directed and less toxic immunosuppression.

One such approach entails the use of pre-transplant donor antigen presentation under the cover novel immunosuppressive strategies that minimize sensitization and promote donor specific unresponsiveness. There are extensive animal studies on the potential

"tolerogenic" effects of donor specific blood transfusions[61]. Clinically, in the pre-cyclosporine era random and donor specific blood transfusions (DST) were shown to have a beneficial effect on graft outcome in adults and children but their effect has diminished in recent years[62-66]. The benefit of DST has been attributed to different mechanisms[61, 67, 68]. DST protocols have ranged from up to 3 transfusions delivered up to several months pre-transplant to a singe transfusion provided within days or hours of the transplant[69-71]. When donor specific transfusions were initially utilized, the sensitization rate of the potential recipients was about 30%, but that was decreased to about 15%. Eventually, the risk of sensitization by the transfusions appeared to supercede any potential for graft survival improvement and the practice of deliberate donor specific transfusions has been largely abandoned. In a recent multi-center study funded by NIH, non-HLA identical living donor kidney transplant recipients were randomized to control or to receive DST 24 hours prior to transplantation and 7-10 days post-transplant[72]. Patients were treated with conventional immunosuppression. Although there was no difference in graft survival at 1 and 2 years, immunologic hyporesponsiveness as determined by the MLR occurred more frequently (18%) in transfused patients compared to controls (3%). Interestingly, transfused patients with more HLA class 1 mismatches had higher incidence of acute rejection, possibly related to sensitization. Another finding in that study was the demonstration that patients who received blood stored for more than 3 days had fewer early rejection episodes than recipients of blood stored for fewer than 2 days[72]. One possible explanation is that blood stored for less than 2 days may contain larger numbers of live leukocytes resulting in sensitization. Therefore, the potential of pre-transplant tolerizing protocols by deliberate exposure of the recipient to donor antigen under non-sensitizing conditions remains a desirable goal in transplantation[73]. The use of agents to minimize or eliminate sensitization by DST are necessary before this approach would be acceptable for pediatric recipients.

Another approach would entail the removal of one or more of the current immunosuppressive agents, perhaps by utilizing less toxic ones. As indicated above, steroids and calcineurin inhibitors have the most toxic side effects. There have been multiple studies directed at steroid withdrawal in pediatric renal transplant recipients[9, 27, 31]. The majority of pediatric patients in these studies have not achieved steroid free immunosuppression[30, 32]. In addition, amelioration of steroid side-effects, especially growth retardation, can be achieved by tapering the steroid dose to an every-other-day schedule[17, 18]. CCTPT will soon undertake a controlled steroid withdrawal protocol utilizing sirolimus immunosuppression. As noted above, calcineurin inhibitors have even worse side effects, most notably acute and chronic nephrotoxicity, hypertension and infectious complications, especially PTLD. A recent study demonstrated that cyclosporine could be avoided in adult renal transplant recipients by utilizing IL-2r antibody induction, combined with MMF and steroids[74]. However, the acute rejection rate in cadaver transplant recipients with delayed graft function was high and as such MMF and steroids alone do not provide the necessary immunosuppression to minimize rejection rates. In another study, azathioprine or MMF and steroids were combined with the new immunosuppressive agent, Sirolimus, in adult renal transplant recipients and resulted in excellent short term success, with better renal function than found in patients treated with cyclosporine[75, 76]. The risk of rejection in the Sirolimus treated patients in both studies was equivalent to the cyclosporine treated patients. The major side effects

in the Sirolimus-treated patients included thrombocytopenia, hypercholesterolemia, hypertriglyceridemia and diarrhea.

Sirolimus is the first clinically available macrolide immunosuppressive drug of the family that are collectively known as the TOR-inhibitors. Sirolimus inhibits both T-cell and T-cell independent B cell effector functions[77, 78]. Sirolimus inhibits T cell signal transduction and may provide T cell costimulation blockade[79]. Importantly, because it does not inhibit IL-2 secretion (calcineurin inhibitors inhibit IL-2 secretion) Sirolimus may be permissive of antigen independent cell death and thus may promote hyporesponsiveness, especially when combined with administration of donor antigen such as DST[80-82]. Sirolimus has been demonstrated to provide potent immunosuppression in recent clinical trials of kidney transplantation[83]. In these studies, Sirolimus was provided in without induction therapy, but in conjunction with cyclosporine. However, recent studies from Europe have demonstrated that Sirolimus can be combined with MMF and steroids to provide excellent graft survival in the absence of calcineurin inhibitors[75, 76]. Importantly, patients receiving this immunosuppression had better renal function than patients who were treated with cyclosporine. Such a strategy would be greatly desirable in pediatric renal transplant studies[84]. There are also preliminary results that suggest that the immunosuppressive macrolides have an in vitro inhibitory effect on growth of PTLD-like EBV + B cell lines, suggesting that they might be preferentially used in transplant recipients at risk of developing PTLD[85]. We hypothesize that immunosuppression IL-2r antibody, Sirolimus, MMF and alternate day steroids will provide comparably graft survival for living donor recipients, compared to current immunosuppression, but with reduced complications of calcineurin inhibitors.

Thus we have developed this protocol which entails the avoidance of calcineurin inhibitors for immunosuppression. The advantages of avoiding these medications are several. Importantly, the calcineurin inhibitors have been implicated in the pathogenesis of PTLD and avoidance may lessen the risk of this serious and frequently fatal complication. Importantly, both cyclosporine and tacrolimus cause acute and chronic nephrotoxicity which may shorten overall graft half-lives substantially. Furthermore they also have other serious side effects, specifically the enhancement of post transplant diabetes, neurotoxicity and, especially in the case of cyclosporine, cosmetic changes which may encourage non-compliance. Since the use of Sirolimus for immunosuppression has not associated with any of these complications, we expect that transplantation in children treated with this protocol will have fewer complications and may have longer half-lives.

Furthermore, this protocol is the first part of a two step proposal to use pre-transplant DST, combined with sirolimus and MMF to prevent sensitization and promote donor specific hyporesponsiveness. Pre-clinical studies have demonstrated that deliberate transfusion combined with sirolimus immunosuppression results in extremely low levels of sensitization. We hypothesize that the DST administered under these conditions will permit substantial reduction of long term immunization, including possible steroid withdrawal. If achieved, the resulting protocol will provide substantial reduction of post transplant morbidity and may lead to even more enhanced long-term graft survival. Step one, the current protocol, will provide data on the safety and efficacy of the calcineurin sparing protocol in children. The clinical studies will be coupled by intense immunologic monitoring which are necessary to better understand the graft function, graft morphology

and the immunologic status of the recipient. One of the major aims of this trial is to develop a battery of assays that can be utilized to provide a better understanding of the immunologic status of the recipient. These studies are essential for step two protocols which involve DST as a tolerogenic regimen with plans to further taper immunosuppression.

As immunosuppression is reduced and, in some cases discontinued, careful immunologic monitoring is essential to prevent or identify rejection at its earliest stages. Previous results for our program and others have established the safety and value of surveillance graft biopsies[86-88]. We propose to extend these preliminary results by evaluating even more sensitive assays of recipient anti-donor reactivity. This will be the first study of immune function in transplant recipients not taking calcineurin inhibitors. In addition to immunologic monitoring, the protocol biopsies, blood and urine obtained at the time of transplant and several additional times throughout the first year will be analyzed by genomic methods to determine differences in gene expression post transplantation.

2.0   STUDY OBJECTIVES

2.1   Primary Objective:

To determine whether a protocol without calcineurin-inhibitors can provide efficacy as good as or better than current standard immunosuppressive protocols with fewer adverse effects, especially hypertension, serious infections and chronic nephrotoxicity. Specifically, this is an uncontrolled pilot assessment of freedom from rejection in the first post-transplant year, using historical rejection rates as a comparison.

2.2   Secondary Objective:

To determine whether the immune inhibition resulting from this protocol can be detected by sensitive and specific assays, including intragraft and peripheral monitoring, for expression patterns of activation and effector function markers. These studies are directed to understanding the mechanisms of action of this immunosuppression and to develop a set of surrogate markers of allograft rejection or hyporesponsiveness in recipients of renal transplants.

3.0   ELIGIBILITY

Pediatric recipients of living donor protocols will be eligible for entry into the first phase of the protocol. Recipients of HLA identical living donor grafts will not be eligible since they are at very low risk of rejection and frequently do not receive calcineurin inhibitors for immunosuppression. Recipients of cadaver donor grafts will not be entered into the first phase because they are at higher risk for rejection episodes and the safety of avoiding calcineurin inhibitors in that setting is unknown. Pediatric is defined as a recipient 21 years of age and under. Centers may exclude potential recipients with focal segmental glomerulosclerosis or oxalosis if the potential for recurrence of the original disease is felt

to be too high. In those settings, changes of immunosuppression are warranted and the value of an experimental protocol seems low. Potential recipients who have failed two or more previous renal transplants will not be eligible. Potential recipients with familial abnormalities of lipid metabolism or levels will not be eligible.

4.0 **METHODS/TREATMENT PROTOCOLS**

4.1 Transplant Protocol

4.1.1 LAB WORK:

CBC with differential and platelets - immediately post-op; evening of surgery; daily thereafter.

Creatinine, BUN every 12 hours post operatively until stable, then daily until discharge.

Liver Function Tests (enzymes) immediately pre-transplant and weekly afterwards.

Fasting cholesterol and triglycerides Monday, Wednesday and Friday until discharge.

Renal Scan and Renal Ultrasounds - first post-op day and PRN.

Serum Sirolimus levels sent daily for the first week, then Monday, Wednesday and Friday until discharge.

4.1.2 IMMUNOSUPPRESSION:

ANTIBODY INDUCTION PROTOCOL:
Dacluzimab (Zenapax) 1 mg/Kg/dose IV for five doses Weeks 0, 2, 4, 6, and 8.

METHYLPREDNISOLONE/PREDNISONE:
Day 0, methylprednisolone 10 mg/kg IV administered in OR.
Day 1, methylprednisolone 10 mg/kg IV.
Days 2 through 180, See Appendix A for total daily dose.
        Note: doses on days 2 - 9 is divided BID
Days 180+, taper prednisone to 0.15 mg/kg/day administered on
        alternate days (max 15 mg q.o.d.) at the discretion of the investigator.

MYCOPHENOLATE MOFETIL (CELLCEPT):
Cellcept: 1200 mg/m^2/day IV or PO, divided BID, TID or QID (the maximum dose
        will be 2 G/day).
Dose should be reduced if WBC < 3000/mm^3 and withheld if WBC < 2000/mm^3.

SIROLIMUS (RAPAMUNE):
First dose: 10 mg/m^2 given as a single dose on Pre-transplant
                        Day 1.

<u>Subsequent Doses:</u>   3 mg/m^2 BID.
Doses are adjusted to maintain steady state blood level (trough) of:
   20-25 ng/mL for the 1st two months
   20 ng/mL for Months 3 through 6
   15 ng/mL thereafter

4.1.3   INFECTION PROPHYLAXIS:

<u>BACTRIM</u>
At resolution of post-operative ileus, begin Bactrim 10 mg TMP/kg (maximum dose 160 mg TMP) three times a week for one year.

<u>GANCYCLOVIR</u>
Infection prophylaxis for CMV and/or EBV will be provided except for donor/recipient pairs who are antibody negative for both CMV and EBV. Oral gancyclovir will be provided at doses noted below, beginning 3 days after transplantation and continuing until 6 months post-transplantation. If patients receive anti-lymphocyte preparations (including ATGAM, OKT3 or Thymoglobulin) for any reason, the gancyclovir will be provided via intravenous route during that treatment and will be continued for at least one month after the completion of treatment.

   Gancyclovir doses:
      For patients > 50 kg: 1 Gram PO TID
      For patients < 50 kg: 500 mg/m^2 TID

   Dose reduction for decreased GFR:
      40-50 mL/min/1.73m^2: Reduce by 50% provided TID
      25-40 mL/min/1.73m^2: Reduce by 50% provided BID
      < 25 mL/min/1.73m^2: Reduce by 75% provided BID

4.1.4   OTHER TREATMENTS

<u>LIPITOR</u>
Treatment with Lipitor will begin if fasting cholesterol > 200 mg/dL is consistently observed. If receiving maximal anti-cholesterol therapy, cholesterol levels > 350 mg/dL is consistently observed, continuation of the immunosuppressive protocol is at the investigator's discretion. If cholesterol levels exceed 600 mg/dL consistently, then calcineurin inhibitor therapy should be initiated without the use of Sirolimus.

4.1.5   TREATMENT OF REJECTION

All Patients who are suspected of having a rejection episode will have a graft biopsy performed, if at all possible, before beginning treatment for rejection.

First acute rejection will be treated with Solu-Medrol, 10 mg/kg, given as a bolus

injection daily for 3 consecutive days.

Second rejection (at the discretion of the transplant center) or severe rejection (Banff Grade 3), will be treated with antibody therapy.

After a severe rejection or after the second rejection, the Mycophenolate will be discontinued and Tacrolimus will be added to the Prednisone and Sirolimus immunosuppression. Tacrolimus will be given daily and will be titrated to a trough whole blood level of 10-20 ng/mL.

4.1.6   MODIFICATIONS

At the discretion of the local investigator, immunosuppression protocols may be modified or discontinued if there are signs or reactions to any of the medications or if there is a serious infection. Sirolimus doses may be modified at the discretion of the study center for hyperlipidemia, thrombocytopenia or for any other side effect thought to be related to the drug. Both MMF and Sirolimus can be associated with anemia, leukopenia and thrombocytopenia. If bone marrow suppression occurs, MMF doses will be adjusted in response to these problems according to center-specific protocols. If anemia (hematocrit < 20) or thrombocytopenia (platelet count < 100,000/mm^3) seem to be related to Sirolimus, the doses should be reduced to reduce the level to 15-20 ng/mL in the first 2 months or 12-15 ng/mL thereafter. If the platelet count falls below 30,000/mm^3, Sirolimus should be discontinued and a calcineurin inhibitor should be begun. Sirolimus will be discontinued at any time if the patient or the family requests discontinuation from the study, or if there is a serious emergency such as a life-threatening infection. MMF doses will be adjusted for leukopenia or gastrointestinal toxicity. If the patient is unable to tolerate MMF, because of gastrointestinal side effects, azathioprine will be substituted for MMF. Preliminary studies in adults demonstrated that the combination of Prednisone-Rapamycin-Azathiopriine provided equivalent outcome as Prednisone-Cyclosporine-Azathioprine[76]; thus this combination should provide no added risk to the MMF intolerant individual. If the patient is unable to tolerate either MMF or azathioprine because of bone marrow toxicity, the patient will be withdrawn from the study and Cyclosproine or Tacrolimus will be substituted for MMF. We are aware that an independent safety committee will be monitoring study outcomes and events and make decisions on study discontinuation.

5.0   **SCHEDULE OF EVENTS**

Patients will be evaluated prior to transplantation by the center's usual protocol. All appropriate vaccinations, including varicella vaccine, will be provided before transplantation. Pre-transplant recipient evaluations will be performed within 72 hours prior to transplantation and will include a complete history and physical examination, CBC, liver function tests, antibodies for CMV, EBV, HIV, HbsAG, and HCV. Beginning on the day of transplant (Day 0), blood will be obtained for Sirolimus assays. Sirolimus assays will be performed by HPLC at Children's Hospital Boston or Children's Hospital of Philadelphia laboratories. All locally performed trough levels, including those evaluated

during non-study visits, will be reported to The EMMES Corporation for analysis. For all scheduled visits, SRL troughs will be sent to Quest Diagnostics for evaluation. This will be in addition to the samples evaluated locally. Mycophenolate Mofeteil (MMF) levels will be obtained weekly for the first three months post transplant. We plan to follow the patients for 36 months. During this period patients will have repeated clinical/laboratory evaluations. There are extensive experimental data and some clinical studies indicating that the status of the graft before transplantation may determine or be an important determinant of early and late outcome. Therefore, we will obtain prospective clinical data by biopsy at the time of transplant. Thus, a transplant biopsy will be performed at the time of the transplant and at 3, 6, and 12 months post transplantation and at times when a rejection is suspected. Biopsies will be evaluated locally and will be sent to the central lab to be assessed by two independent pathologists for acute and chronic rejection utilizing the Banff criteria. A radionuclide GFR will also be done at the same time points, and at 24 and 36 months. Clinical safety will be monitored through routine physical examinations and appropriate laboratory assessments. Infections, especially opportunistic infections will be assessed. Monitoring for development of CMV and EBV will be undertaken. The follow-up schedule is described in the following tables.

TABLE 1: TESTS/EVALUATIONS IN THE FIRST MONTH

Tests/Evaluation	Pre-Transplant*	Day 0**	Day 3	Day 10	Day 14	Day 28
Medical History	X					
Demographics	X					
Physical Exam	X			X	X	X
Vital Signs	X	X	X	X	X	X
CBC w/Differential	X	X	X	X	X	X
Blood Chemistry	X	X	X	X	X	X
Lipid Levels	X		X	X	X	X
Urinalysis	X	X	X	X	X	X
Urine Protein/creatinine	X	X	X	X	X	X
S. Pregnancy Test	X					X
PRA	X					
Informed Consent	X					
HIV Ab	X					
CMV and EBV Ab	X					
HbsAg and HCV Ab	X					
GFR						X

*Within 72 hours prior to transplant
**Within 24 hours after transplant

TABLE 2: TESTS/EVALUATIONS DURING FIRST YEAR

Test/Evaluation	Week 6	Month 2	Month 3	Month 6	Month 12
Physical Examination	X	X	X	X	X
CBC w/Differential and Chemistry	X	X	X	X	X
Lipid Levels	X	X	X	X	X
Urinalysis	X	X	X	X	X
Urine Protein/creatinine	X	X	X	X	X
GFR			X	X	X

TABLE 3: TESTS/EVALUATIONS AFTER THE FIRST YEAR

Test/Evaluation	Month 18	Month 24	Month 36
Physical Examination	X	X	X
CBC w/Differential and Chemistry	X	X	X
Lipid Levels	X	X	X
Urinalysis	X	X	X
Urine Protein/creatinine	X	X	X
GFR		X	X

TABLE 4A: SPECIMENS SUBMISSION SCHEDULE

Study Day Visit Number	Screening* 000	Day 0 001	Day 3 002	Day 10 003	Wk 2 004	Day 28 005	Wk6 006	Mo2 007	Mo3 008	Mo6 009	Mo12 010	Mo18 011	Mo24 012	Mo36 013	REJ**
PCR – Biopsy		X							X	X	X				X
PCR – Blood		X	X	X	X	X	X	X	X	X	X				X
PCR – Urine		X	X	X	X	X	X	X	X	X	X				X
Gene CHIP – Frozen Blood		X							X	X	X				X
Gene CHIP – Urine		X	X	X	X	X	X	X	X	X	X				X
Immunohistology Biopsy		X							X	X	X				X
Alloantibodies – Serum	X								X	X	X				X
Cellular Immune Monitoring – Whole Blood	X								X	X	X				X

*Within 72 hours prior to transplant
**Collect for all suspected rejection episodes

TABLE 4B: SPECIMENS

STUDY	Specimen Type	Section Of Protocol*
PCR Gene Expression	Biopsy (1/2 Core)	5.1
PCR Gene Expression	Blood (2.5mL)	5.6
PCR Gene Expression	Urine (100mL)	5.3
Gene CHIP	Frozen Blood (5mL)	5.2
Gene CHIP	Urine (100mL)	5.3
Immunohistology	Biopsy (1/2 Core)	7.0
Alloantibodies	Serum (5-6mL of blood)	5.5
Cellular Immune Monitoring	Whole Blood (40mL for pts > 6 yrs old) (20mL for pts < 6 yrs old)	5.4

* Process described in identified section of protocol

TABLE 4C: TESTS/EVALUATIONS REQUIRED

	Screening 000	Day 0 001	Day 3 002	Day 10 003	Wk 2 004	Day 28 005	Wk6 006	Mo2 007	Mo3 008	Mo6 009	Mo12 010	Mo18 011	Mo24 012	Mo36 013	REJ
MMF Levels ** UPenn															
MMF PK- UPenn						X			X						
SRL Levels- Quest		X	X	X	X	X	X	X	X	X	X	X	X	X	X
SRL PK- Taylor						X			X						
Local SRL ***		X	X	X	X	X	X	X	X	X	X	X	X	X	X

** To be collected every week up to, but not including month 3
***To be collected as indicated and as needed to obtain target dose levels

5.1   Handling, Storage, and Shipment of Renal Biopsy Samples

Two cores will be obtained at the time of biopsy. One core will remain at the center and sent to the local pathologist. The remaining core of an 18g percutaineous biopsy will be cut in half at the **bedside**. One half of each core will be submitted to each of the central labs for future analysis.

One half of one of the cores will be submitted to the central laboratory for special staining and interpretation. This specimen will be placed in a labeled cryotube, snap-frozen in liquid nitrogen and stored in a -70° C freezer until shipment. Note: Strict adherence to these instructions is absolutely essential. Tissue morphology and antigenicity remain stable if snap frozen specimens are stored at -70° C. Tissue not handled properly will be highly subject to degradation.

This specimen is to sent to:

> Wayne Hancock, M.D., PhD
> Pathology, 807B Abramson Research Center
> The Children's Hospital of Philadelphia
> 3615 Civic Center Blvd
> Philadelphia, PA 19104-4318
> Phone: 215-590-8709
> Fax: 215-590-7384
> Contact: hancock@email.chop.edu

One half of the other core will be **immediately** stored in a -70°C freezer until shipment. All cryotubes should be labeled with identifying patient and institution information prior to initial freezing. **Note: Strict adherence to these instructions is absolutely essential. Tissue remains stable if snap frozen specimen is stored at -70° Celsius. The samples should not be permitted to defrost at any time. Samples not handled per this procedure are highly subject to degradation.**   This specimen is sent to:

> Terry Strom, M.D.
> Division of Clinical Immunology
> Beth Israel Hospital
> Research North, Room 380
> 99 Brookline Avenue
> Boston, MA 02215
> Phone: (617) 632-0150
> Fax: (617) 632-0160
> Contact: tstrom@caregroup.harvard.edu

Specimens must be shipped Monday through Thursday via same day courier i.e., Federal Express. Biopsies should be shipped when the institution has accumulated between 6 and 18 samples. Specimens are to be shipped in a Styrofoam container on dry ice, to assure continuous freezing. Wear gloves when handling specimens and

transfer them quickly from the -70° Celsius freezer into the middle of the dry ice. The specimens must be surrounded with a minimum of 10 cm of dry ice on all sides of the shipping container. Secure the Styrofoam container lid with duct tape to insure a well-fitting seal. Please be certain to include with the specimen the transplant recipient's initials, CCTPT identification number, date the specimen was obtained and the name of the sending institution.

Schedule the arrival of the specimens by calling the laboratory. Provide the recipient with the following information: name of the carrier, air bill number, expected date of shipment arrival, the number of pieces in the shipment, and the name and telephone number of the contact person at the sending institution to notify if the shipment is not received. Every attempt should be made to have the specimens delivered Monday through Thursday. If it is absolutely necessary to ship specimens on a weekend, follow the procedures outlined above. Specimens shipped on weekends should be scheduled such that they arrive at the laboratory no later than 5:00 p.m. Eastern Time.

5.2   Collection, Processing, Storage, and Shipment of Frozen Whole Blood Specimens

Use only special plastic EDTA-LAVENDER TOP vacutainer tubes. DO NOT USE GLASS VACUTAINER TUBES AS THEY CAN BREAK IN STORAGE AT -70°C. 5-6 mL of blood should be draw into each vacutainer tube. Invert the tube 1-2 times. Each tube should be labeled with the patient's initials, ID and date the blood is drawn. Store the blood specimen in a -70°C freezer within 30 minutes after drawing the blood specimen. Fill in the required information on the Specimen Submission Form.

When shipping the sample, always arrange the shipment by same/next day courier i.e., Federal Express to arrive Monday through Thursday. At the time of the shipment, fill an undamaged Styrofoam container with dry ice. Transfer samples quickly from the -70°C freezer into the middle of the dry ice. Cover them with more dry ice so that the samples are completely surrounded by at least 10 cm of dry ice on all sides. Cover the Styrofoam container with a well fitting cover and seal the box with the duct tape.

Call (617) 632-0150 one day prior to mailing the specimen. Ship the Styrofoam box and copies of the Specimen Submission Form for each specimen to Dr. Strom at the address listed above.

5.3   Collection, Processing, Storage and Shipment of Urine

Urine should be collected prior to biopsy when applicable. Optimally 100mL of urine are to be collected (but no less then 50mL). Centrifuge the urine at 2,000g at room temperature for 30 minutes in sterile disposable tubes. Discard the supernatant completely. There should not be any trace of urine left in the tube. Resuspend the pellet in 1.0mL of phosphate buffered saline (PBS). Make sure that any sediment on the sides of the tube is also collected. Transfer to a NUNC cryotube. Centrifuge at 16,000g for 4 minutes at room temperature. Discard the supernatant gently and completely without disturbing the cell pellet. Add three drops (150 μL) of RNA later to the pellet and close the cap tightly. Gently tap the lower portion of the tube with your finger to mix the

cell pellet with the RNA later. Label each NUNC cryotube with the patient's initials, identification number, the date, and the initial amount of urine collected. Also complete the required information on the Specimen Submission Form. Centrifuge the tube at 16,000g for 15 seconds.

Hold the tube with the urine pellet in the vapor phase of liquid nitrogen to snap freeze. Place the tube deep into the dry ice box to prevent thawing of the urine pellet. Transport the dry ice box to a -70°C freezer. Remove the tube from the dry ice box and immediately place it in the freezer.

When shipping the sample, always arrange the shipment by same/next day courier i.e., Federal Express to arrive Monday through Thursday. At the time of the shipment, fill an undamaged Styrofoam container with dry ice. Transfer samples quickly from the -70°C freezer into the middle of the dry ice. Cover them with more dry ice so that the samples are completely surrounded by at least 10 cm of dry ice on all sides. Cover the Styrofoam container with a well fitting cover and seal the box with the duct tape.

Contact Ms. Ann Seton at the IMMUNOGENETICS AND TRANSPLANTATION CENTER at (212) 772-6700 one day prior to mailing the specimen. Ship the Styrofoam box and the Specimen Submission Forms for each specimen to:

> Dr. M. Suthanthiran, MD
> Immunogenetics and Transplantation Center
> 430 East 71st Street
> New York, NY 10021
> Phone: (212) 772-6700 (Ann Seton)
> Phone: (212) 746-4430 (Sue Campus)

5.4 Collection, Processing, Storage, and Shipment of Mononuclear Cell/Whole Blood Specimens

40 mL of blood from each individual should be drawn into four 10 mL green top tubes (Sodium Heparin tubes). (For children less than 6 years of age, please obtain 20 mL into each of two 10mL tubes) Each tube should be labeled with the patient's initials, ID and date the blood is drawn. Ship the blood same day at room temperature such that the blood is received within 24 hrs of the time of the blood draw.

When shipping the sample, always arrange the shipment by same/next day courier i.e., Federal Express to arrive Monday through Thursday. Please use a Styrofoam container. Cover the specimens with packing material so that the samples are completely surrounded on all sides. Cover the Styrofoam container with a well fitting cover and seal the box with the duct tape.

Call (617) 247-5179 one day prior to mailing the specimen. Ship the Styrofoam box and copies of the Specimen Submission Form for each specimen to:

Dr David M. Briscoe
Transplant Immunology Laboratory
Children's Hospital
21-27 Burlington Ave,
4th Floor. Room 465
Boston, MA 02215

5.5   Collection, Processing, Storage and Shipment of Serum

10 mL of blood from each individual should be drawn into a red top tube. KINDLY DO NOT USE RED TOP TUBE WITH ANY GEL INSIDE. Label each of 3 cryotubes with the patient's initials, identification number, and the date the specimen was drawn. Centrifuge the red top in a table top centrifuge at 1000g for 15 minutes. Then remove the clear serum from the tube and transfer the serum into 3 pre-labeled cryotubes. Store the specimens at -70°C freezer and complete the required information on the Specimen Submission Form.

When shipping the sample, always arrange the shipment by same/next day courier i.e., Federal Express to arrive Monday through Thursday. At the time of the shipment, fill an undamaged Styrofoam container with dry ice. Transfer samples quickly from the -70°C freezer into the middle of the dry ice. Cover them with more dry ice so that the samples are completely surrounded by at least 10 cm of dry ice on all sides. Cover the Styrofoam container with a well fitting cover and seal the box with the duct tape.

Call (617) 247-5179 one day prior to mailing the specimen. Ship the Styrofoam box and copies of the Specimen Submission Form for each specimen to

Dr David M. Briscoe
Transplant Immunology Laboratory
Children's Hospital
21-27 Burlington Ave,
4th Floor. Room 465
Boston, MA 02215

5.6   Collection, Processing, Storage, and Shipment of Blood Specimen for RT-PCR Studies

This specimen will be collected as specified in Table 4 of the protocol. The CCC will supply each center with PAXgene Blood RNA Tubes to be used for these samples. These tubes must be stored at room temperature (18-25°C) and labeled with the subject's initials, ID number, site number, and the date the blood is drawn. Using a standard blood collection set, collect blood directly into the PAXgene Blood RNA Tube using your institution's recommended procedure for standard venipuncture technique. Hold the PAXgene RNA Tube vertically below the blood donor's arm during the

collection. One (1) 2.5 mL tube should be filled. Allow at least 10 seconds for a complete blood draw to take place. Ensure that the blood has stopped flowing into the tube before removing the tube from the holder. Gently invert the PAXgene Blood RNA tube 8-10 times. The tube should be kept at ambient temperature for 6 - 24 hours to allow it to complex, then it should be frozen at -70 degrees and batch shipped frozen to your specified lab in dry ice.

6.0   **ADVERSE EXPERIENCES**

6.1   Reporting Requirements

Sites should report Adverse Experiences to the Clinical Coordinating Center as described below:

Adverse Experience	Reporting Requirements
Deaths and life threatening events and other serious adverse events	Telephone: (301) 251-1161 and Submit Adverse Event Form within 24 hours

6.2   Serious Adverse Events (SAEs)

All Serious Adverse Events must be reported to the Clinical Coordinating Center within 24 hours of the event.

A **Serious Adverse Event** is an event that results in any of the following outcomes:

- ♦ Death
- ♦ A life threatening adverse drug experience that places the patient, in the view of the investigator, at immediate risk of death from the adverse event.
- ♦ Requires or prolongs inpatient hospitalization.
- ♦ Results in persistent or significant disability/incapacity. This criterion applies if the "disability" caused by the reported adverse event results in a substantial disruption of a person's ability to conduct normal life functions.
- ♦ Causes a congenital anomaly/birth defect.
- ♦ Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the patient and may require medical or surgical intervention to prevent one of these outcomes.

7.0   **MOLECULAR MONITORING**

PCR GENE EXPRESSION IN ALLOGRAFT BIOPSY:

We will test kidney biopsy material at the four time points (Immediately following perfusion of the graft and at 3, 6, 12 months following transplant) for expression profiling using cDNA microassay analysis and for quantitative mRNA assessment by real-time PCR using TAQMAN analysis of a specific set of genes (listed below) that have been correlated with acute or chronic rejection.

> Cytotoxic lymphocyte genes: Perforin, Granzyme B, FasL
> Th1/Th2 cytokines: IL-2, IFN-gamma, IL-4, IL-10
> Chemokine and chemokine receptor genes: CXCR3, IP-10, CCR4, MDC
> Adhesion Molecules: VCAM-1
> TGF-beta 1
> CMV
> Constitutive gene expression: GAPDH, Cyclophylin B

PCR GENE EXPRESSION MONITORING:

Surveillance studies on PBLs and urinary lymphocytes will be performed at the time of transplant and during the first two weeks, at the time of Zenapx treatment at 2 weeks, 4 weeks, 6 weeks, 2 months, and at the time of surveillance biopsies at 3 months, 6 months, 12 months and at time of biopsies for clinical events. The specimens will be assessed by real time PCR and by gene chip. The initial list of mRNA specimens includes the following: Perforin, Granzyme B, FasL, IL-2, IL-4, IFN-gamma, IL-10, IL-15, TGF-beta1, Cyclophylin B, CMV, GAPDH.

IMMUNOHISTOLOGY:

As standard morphologic assessment will be undertaken by pathology staff at the host institution, the portion of each renal biopsy supplied snap-frozen will be sectioned on a cryostat for immunopathology. As received, samples will be encoded and subsequently evaluated in a blinded manner. Slides for one biopsy will be stained by H&E for orientation and the remaining sections will be evaluated by immunoperoxidase for the presence of activation molecules as defined by mAbs. We plan to examine the following markers: Ig control, CD45 (baseline leukocyte marker for image analysis) and class II MHC, B7.1 and B7.2, CD50L, and CD40, IFN-gamma, TNF-alpha, IL-4 and IL-2R, CXCR3 and ligands (IP-10, Mig), CCR4 and ligands (TARC, MDC), TUNEL+ (cells undergoing apoptosis, anti-human IgG, Protective genes (Bcl-2, Bcl-xL, A20 and HO-1), and VCAM-1. Labeling will be assessed by computer assisted image analysis (IP-Lab spectrum software, microscopic fields digitized using a Leaf-Microlumina camera attached to a Macintosh G3 computer). A quantitative system for recording results from each encoded biopsy will be maintained. Digitized images will also be available to other investigators in the study via internet access to our server, if such access is desired (password-protected). The results from these studies will be compared with intragraft RT-PCR studies above.

DETECTION OF ALLOANTIBODY:

Alloantibody will be detected in sera of transplant recipients using an ELISA technique, (PRA_STAT, SangStat, Menlo Park, CA). This solid phase immuno assay utilizes soluble HLA antigens captured on an ELISA microplate. The technique allows for the testing of serum against 48 different HLA antigens, including class I and class II alloantigens. Following incubation of patients serum with HLA coated microwells, the presence of antibody is revealed using a peroxidase-conjugate anti-human IgG antibody. The ELISA results are analyzed in a standard ELISA ready (Molecular Devices) at 495 nm.

CELLULAR IMMUNE MONITORING:

Responses to donor cells and HLA peptides: Patients will be studied for PBL reactivity to donor-derived peptides and donor cells (MLR) at the time intervals depicted in Table 4. We plan to synthesize a total of approximately 30 peptides corresponding to the beta-chain hypervariable domain of approximately 10 HLA-DR molecules. We anticipate that this will cover most if not all potential DR incompatibilities in our recipients of cadaver as well as live organs. The methods of measuring peptide alloreactivity have been previously published by our group. For live donor recipients we plan to also test reactivity to donor cells in the standard one way MLR, as previously reported by Reinsmoen. Patients will be entered into an observational study to monitor PBL reactivity to mismatched DR antigen/s of the graft donor. Specificity will be tested by using third party stimulator cells from normal volunteers (for the MLR) or non-mismatched HLA-DR peptides (for peptide alloreactivity).

Generation and Characterization of Allopepide-Specific T Cell Lines: Recent data from our laboratory show that MHC allopeptide-specific T-cell lines/clones from animals with rejection are of the Th1 phenotype, while those from tolerant animals are of the Th2 phenotype. At 1 year post-transplant we plan to generate short-term T cell lines against donor HLA-DR peptides after 3 stimulations in the culture. The lines will be analyzed by flow cytometry to determine phenotype and by RT-PCR to define their TCR Vβ expression. We will also define their activation pattern, including cytokine profile (Th1 versus Th2) and ability to secrete the fibrogenic growth factor (TGF-β), by using specific human standard ELISA kits on culture supernatants. Because of the recent data indicating the importance of chemokines and chemokine receptors in characterizing T helper cell phenotype, and their potential relevance to the processes of acute and chronic rejection and tolerance (see preliminary results above), particular emphasis will be placed on analyzing expression of these molecules by RNAse protection assays. The emphasis will be correlated with the immunohistological studies performed on transplant biopsies at 1 year (above). The cell lines will then be used for basic studies in SCID mice to study the role of Th1 and Th2 cell lines in promoting rejection or regulation of alloimmune responses in vivo.

Assessment of Apoptosis: We will assess the number of alloreactive lymphocytes and undergoing apoptosis in treated and untreated patients. T cell responses and tolerance induction are controlled by a balance between the survival and expansion of alloreactive T cells on the one hand, and the induction of apoptosis on the other. The stimuli that promote the survival, proliferation and differentiation of T cells includes co-stimulators, growth factors and cytokines. Following initial activation the vast majority of cells undergo a process of activation induced cell death (AICD) and a small number of lymphocytes survive and differentiate into effector and memory T cells. It is evident that the cytokine IL-2 is critical for the development of AICD, which is dependent on interactions among FasL and Fas. Il-2 dependent induction of FasL and AICD is thought to be critical for peripheral tolerance induction. Fas signaling in activated T cells induces apoptosis following stimulation or re-stimulation specific antigen. This apoptosis pathway is thought to function predominantly for the maintenance of self-tolerance and in the eradication of "non-specific" activated cells. However, there is evidence to suggest that IL-2 also regulates tolerance by additional mechanisms. IL-2-regulated expression of anti-apoptotic proteins Bcl2/Bclx1 may be important mediators of long term memory. The Bcl2/Bclx1 proteins inhibit the activation of select death pathways but have no effect on Fas-dependent death (AICD). IL-2 signals as well as co-stimulation (e.g. CD28 stimulation) are potent for the induction of the Bcl2/Bclx1 family of molecules, while IL-2 is specific for the induction of the Fas-dependent AICD death pathway. In our studies blockade of IL-2 may limit T cell proliferation and AICD, whereas Rapamycin in the absence of calcineurin inhibition may promote apoptosis of alloreactive memory T cells by limiting IL-2-dependent induction of Bcl2/Bclx1 proteins. This mechanism has been demonstrated in animals when Rapamycin has been recently demonstrated to inhibit the persistence of alloreactive T cells and to promote "tolerance" by inhibiting the induction of anti-apoptotic genes. Thus, since our patients will receive Rapamycin in a "low IL-2 state" (IL-2R blockade) it is possible that there will be changes in the level FasL-Fas dependent mechanisms (AICD); and/or that Rapamycin will augment tolerance via inhibition of anti-apoptotic gene expression. We will evaluate both possibilities. The level of apoptosis will be determined in CD4+ and in CD8+ T cells from untreated or treated patients enrolled in the study. We will correlate apoptosis with the expression of FasL and Bcl2/Bclx1 on lymphocytes and with the presence of AICD. Lymphocyte subsets will be purified by negative selection and will be cultured at a density of $(1x10^6)$/mL for 24 hours alone or with 50U/mL of IL-2 in tissue culture wells coated with 1mcg/mL of either anti-CD3 antibody or anti-CD28 antibody (Pharmingen). To assess Fas-dependent apoptosis, cells are first incubated with mouse anti-human Fas antibody (Pharmingen) and then cultured for 24 hours with 50U/mL of IL-2 in the presence of a crosslinking antimouse Ig. In both instances, the cells will be fixed in 2% paraformaldehyde and assayed for apoptosis by propidium iodine staining. The expression of FasL will be assessed on T cells by flow cytometry (FACS). The expression of Bcl2 and Bclx1 proteins will be assessed by intracellular staining and FACS analysis. Briefly cells will be fixed in 2% paraformaldehyde as above and permeabolized with 0.1% Triton X-100 and stained with a directly conjugated anti-Bcl2 antibody. In all Controls will be cells stained with isotype control antibody. We will confirm the expression of Bcl2 protein by Western Blot. All analyses will be also

assessed following reactivation of T cells with donor antigen using readouts as the MLR (direct) or peptide (indirect, as discussed above).

8.0 **STATISTICAL ANALYSIS**

We are proposing that the study be a case series to determine whether the acute rejection rate is acceptable to permit further study. We will select between two hypotheses a true rejection rate of 0.2 and a true rejection rate of 0.4. These values were chosen because the investigators feel that a six-month rejection rate of 0.4 or higher is unacceptable given current treatment regimens and a six-month rejection rate of 0.2 or lower is acceptable given the expected benefits of being free of a calcineurin inhibiting drug. If we have sufficient evidence to conclude that the rate is ≤ 0.2 (or ≥ 0.4) we will terminate the study and act accordingly for further studies. The sequential testing procedure to be used is a truncated extension of the Sequential Probability Ratio Test (SPRT) [89]. A description of the test follows.

The particular restrictions imposed for this study are $\alpha = \beta = 0.06$ with H_O: $\theta_O = 0.2$ and H_a: $\theta_1 = 0.4$ where θ represents the six-month rejection rate. The maximum number of patients enrolled will be 35. The number of patients rejecting before six months is plotted against the total number of patients in the study. The trial will be stopped if a total of 35 patients are put on trial (and observed for six months) or the test statistic falls out of the continuation region defined by two parallel lines with a slope of 0.293 and intercepts of –2.805 and 2.805. If the test statistic crosses the upper bound the null hypothesis will be rejected in favor of the alternative. If the test statistic crosses the lower bound the alternative hypothesis will be rejected in favor of the null. If the trial proceeds to enroll 35 subjects, we would call the trial successful if 10 or fewer rejections were observed (and unsuccessful if 11 or more). The stopping bounds are derived from a closed-ended modification to the sequential probability ratio test. Also, the upper (lower) bound is rounded down (up) to an integer value when the true bound is within 0.1 of that integer value

Translating the above early termination criteria for the upper bound (i.e. termination for too many acute rejection episodes) into tabular form, the following stopping criteria apply:

TABLE 5: STOPPING CRITERIA FOR REJECTION RATE

# of Patients	1-3	4	5-7	8-11	12-14	15-18	19-21	22-24	25-28	29-31	32-34
# of Rejections	NA	4	5	6	7	8	9	10	11	12	13

The usual measures of performance of a SPRT are the error probabilities α and β of rejecting H_O when $\theta=\theta_O$ and of accepting H_O when $\theta=\theta_1$, respectively, and the expected sample size $E(N| \theta)$. The operating characteristics of the test to be used in this protocol are shown in Table 1 below. Because the SPRT is truncated at 35, the realized type I error rate is 0.08 and the type II error rate is 0.12. These operating characteristics were determined theoretically and validated in a simulation study of 100,000 replications.

TABLE 6: OPERATING CHARACTERISTICS SEQUENTIAL TESTING PROCEDURE

True 6 Month Rejection Rate	10%	15%	20%	25%	30%	35%	40%	45%	50%
Probability Reject Null	0.00	0.01	0.08	0.25	0.49	0.73	0.88	0.96	0.99
Mean # Patients	15	19	25	26	26	24	21	17	14

9.0 **APPENDIXES**

APPENDIX A - STEROID TAPER

Total daily dose of prednisone for study days 2 through 180. Doses for children between the given weight classes should be interpolated. Total daily dose for children over 60 kg should use the total daily dose for children weighing 60 kg. All doses administered QD except doses administered on days 2-9.

Day	Weight (kg)										
	10	15	20	25	30	35	40	45	50	55	60
2-3*	20	30	40	50	60	60	60	60	60	60	60
4-6*	17.5	26.5	35	37.5	45	52.5	60	60	60	60	60
7-9*	15	22.5	25	25	30	35	40	45	50	55	60
10-12	12.5	12.5	12.5	12.5	15	17.5	20	22.5	25	27.5	30
13-15	10	12.5	12.5	12.5	15	17.5	20	22.5	25	27.5	30
16-18	7.5	11.25	12.5	12.5	15	17.5	20	22.5	25	27.5	30
19-29	5	7.5	10	12.5	15	17.5	20	22.5	25	27.5	30
30-43	4	6	7.5	9.5	11.5	13.5	15	17	19	21	22.5
44-59	2.5	4	5	6.5	7.5	9	10	11.5	12.5	14	15
60-180	2	3	4	5	6	7	8	9	10	11	12
180+	Taper prednisone to 0.15 mg/kg/day administered on alternate days at the discretion of the investigator (max 15 mg q.o.d.)										

* Prednisone dose divided BID for study days 2 - 9.

APPENDIX B - CN01 PHARMACOKINETICS PROTOCOL

SAMPLING TIMES FOR WHOLE BLOOD SRL AND MMF (MPA)* MONTHS 1 AND 3
POST TRANSPLANT

STUDY PERIOD	Time Of Specimen Following Dose						
	0 Hours**	40 Minutes	1 Hour	2 Hours	3 Hours	8 Hours	12 Hours**
SRL	X$^+$		X	X	X	X	X
MMF$^+$	X$^+$	X	X	X			

* Subject on once-per-day dosing of SRL should also have SRL levels at 16 and 24 hours
** Trough level, just prior to the next dose
+ Fasting Levels

ALL patients will also have trough MMF (MPA) levels drawn weekly, for the first 3 months after transplant, excluded weeks when AUC sampling is performed.

Please note how the SRL dose is given relative to meals at home:

      SRL given with meals at home
      SRL given separate from meals at home

There should be no changes in SRL or MMF doses within 48 hours prior to kinetics. If dose changes are medically necessary, postpone kinetics until $\geq$ 48 hours after changes.

Justification:
Previous studies performed by Dr. Les Shaw utilized MPA levels at 0, 40 minutes and 120 minutes to calculate AUC, with an AUC reference range of 30-60 mg*hr/mL. However, these data are from adults. Drug absorption in children tends to be more erratic than in adults, and therefore an additional sample is being proposed at 60 minutes to avoid missing the peak, which is paramount to calculating an accurate AUC. It is possible that if initial results show that 1-hour MPA levels are lower than the 40-minute levels in ALL subjects, the 1-hour sample may eventually be estimated. However, 30 subjects is not a large sample size, and therefore initial data must be analyzed cautiously prior to extrapolating to larger groups.

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