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ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION, MIXED CHIMERISM AND TOLERANCE IN LIVING RELATED DONOR RENAL ALLOGRAFT RECIPIENTS

Trivedi HL[1], Vanikar AV[2], Modi PR[4], Shah VR[3], Vakil JM[1], Trivedi VB[2], Khemchandani SI[4]

ABBREVIATIONS

CsA	Cyclosporin
Cn	Control group
CMV	Cytomegalovirus
ESRD	End stage renal disease
GVHD	Graft versus host disease
HSCs	Hematopoietic stem cells
LCM	Lymphocytotoxicity cross-matching
LRD	Living related donor
MLHC	Mixed lymphohematopoietic chimerism
PBSC	Peripheral blood stem cell
SCr	Serum creatinine
Tn	Treated group

KEY WORDS

hematopoietic stem cell transplantation, chimerism, tolerance, renal allograft recipients

ABSTRACT

We designed a prospective, randomized control clinical trial to evaluate the efficacy and safety of achieving mixed chimerism associated tolerance protocol in living related donor (LRD) renal allograft recipients.

Patients and Methods: Sixty six consecutive patients divided in two equal groups of 33 patients with end stage renal disease, were enrolled for transplantation after negative lymphocytotoxicity cross-matching (LCM). Both groups (treated (Tn) and control (Cn)) were well-balanced in their clinical, lab parameters and donor HLA match profile. Tn underwent thymic transplantation of donor renal tissue, 2 donor specific transfusions, low intensity conditioning and high dose hematopoietic stem cell transplantation (HSCT) before renal transplantation. Conditioning regimen included low dose target specific irradiation (to abdominal and inguinal lymph nodes, bone marrow(BM) of thoracolumbar vertebrae and part of pelvis on alternate days, 100 rads x 4), anti-T cell antibodies (1.5 mg/kg body weight (BW)), cyclophosphamide (10 mg/kg BW x 2 consecutive days) and cyclosporine (CsA) (3 mg/kg BW/day). Unfractionated HSCT procured from donor marrow was administered in BM, portal and peripheral circulation within 24 hours of achieving CD 4^+/ CD 8^+ T- cell count < 10% of normal. This was supplemented by

1. Department of Nephrology and Clinical Transplantation
2. Department of Pathology, Laboratory Medicine, Transplantation Services and Immunohematology
3. Department of Anaesthesiology and Critical Care
4. Department of Urology and Transplantation Surgery

Institute of Kidney Diseases & Research Centre and Institute of Transplantation Sciences

ADDRESS FOR CORRESPONDENCE
Prof. Hargovind L Trivedi, FRCP (C)
Professor & Director
Institute of Kidney Diseases & Research Centre and Institute of Transplantation Sciences
Civil Hospital Campus, Asarwa, Ahmedabad 380016, Gujarat, India
TEL: 0091 79 2268 5600/01/04/05 FAX: 0091 79 22685454 E mail: ikdrcad1@sancharnet.in

peripherally mobilized stem cells, with mean total dose of 20 x 10^8 cells/kg BW of recipient administered peripherally. Renal transplantation was performed after negative LCM. Donor specific cytotoxic antibodies were eliminated with intravenous immunoglobulins and plasmapheresis before renal transplantation. Mixed chimerism was evaluated before and after transplantation in patients with donors of opposite gender by fluorescent in-site hybridization (FISH) technique. Both groups received CsA and prednisolone for immunosuppression, Cn received mycofenolate mofetil/ Azathioprine in addition. Rejection was treated with standard anti-rejection treatment. Immunosuppression was withdrawn 6 months after renal transplantation in patients with consistent positive chimerism. Clinical tolerance was defined by achieving stable allograft function for more than 100 days without immunosuppression confirmed by allograft biopsy.

Results: Over a mean follow-up of 210 days all the patients in Tn had stable allograft function with mean serum creatinine (SCr) of 1.20 mg/dl, no rejection/ CMV infections/ graft and patient loss. Low level donor-specific cytotoxic antibodies were observed in all of them. CsA toxicity was noted in 10 (30.3 %) patients. Persistent mixed hematopoietic chimerism was noted in all 21 patients irrespective of donor-recipient HLA matching (mean 0.5 % before and 1 ± 0.3 % after transplantation). All 4 patients on drug withdrawal have developed donor-specific tolerance with a mean follow up of 129.8 days. Other Tn patients are in the process of being weaned off immunosuppression. Mean SCr of Cn was 1.45 mg/dl over a mean follow up of 216 days. Acute rejection was observed in 17 (51.5 %) patients, no CMV infection/ patient loss was noted and 1 (3.03 %) graft was lost in Cn. No patient was lost in Cn. No graft Vs host disease was observed in Tn.

Conclusion: We have achieved mixed hematopoietic chimerism associated tolerance with high dose HSCT, intrathymic donor renal tissue transplantation and minimum conditioning without any adverse effects.

INTRODUCTION

Tolerance is a Utopian dream of every transplanter. Donor-specific immunologic tolerance induction would protect an allograft from chronic rejection and allow discontinuation of chronic immunosuppressive therapy to avoid side effects like infections and malignancy. Experimental models have shown that mixed hematopoietic chimerism leads to tolerance. However it is not easy to validify this Medawarian concept in humans. We designed and implemented a protocol to induce mixed lymphohematopoietic chimerism (MLHC) associated tolerance in our living related donor (LRD) renal allograft recipients.

PATIENTS

Sixty six consecutive patients with end stage renal disease (ESRD) at the Institute of Transplantation Sciences, Ahmedabad, India, were enrolled for this prospective randomized, open label parallel control, single center clinical trial in our LRD renal allograft recipients from May 2003 to December, 2003 with their informed consent. Thirty three patients were included in each group: treated (Tn) and control (Cn). Patients in both groups were comparable demographically, etiology of ESRD and donor-recipient HLA match profile (tables 1, 2). The three most common etiologies of ESRD were chronic glomerulonephritis, obstructive uropathy and diabetic nephropathy.

Group (n=66)	Treated (Tn) (n=33)	Control (Cn) (n=33)
Mean follow-up (Days)	372	378
Mean age in years (range)	33 (8 -57)	34 (10 – 56)
Gender (M:F)	30:3	30:3
Average 3rd party infusion	21	20
CMV seropositivity		
Recipients	22 (66.7%)	32 (97%)
Donors	23 (69.7%)	32 (97%)
Etiology of ESRD		
Chronic glomerulonephritis	15 (45.5%)	16 (48.5%)
Obstructive uropathy	8 (24.2%)	7 (21.1%)
Diabetic Nephropathy	5 (15.1%)	4 (12%)
ADPKD	2 (6.2%)	2 (6.2%)
Reflux Nephropathy	1 (3%)	2 (6.2%)
SLE	1 (3%)	1 (3%)
IgA Nephropathy	1 (3%)	1 (3%)

Table 1 Patient Demographics.

Group (n=66)	Treated (Tn) (n=33)	Control (Cn) (n=33)
0/6	5 (15.2%)	6 (18.2%)
1/6	10 (30.3%)	11 (33.3%)
2/6	3 (9.1%)	3 (9.1%)
3/6	12 (36.4%)	12 (36.4%)
4/6	1 (3%)	1 (3%)
5/6	1 (3%)	0
6/6	1 (3%)	0

Table 2 Donor Recipient HLA Match Profile.

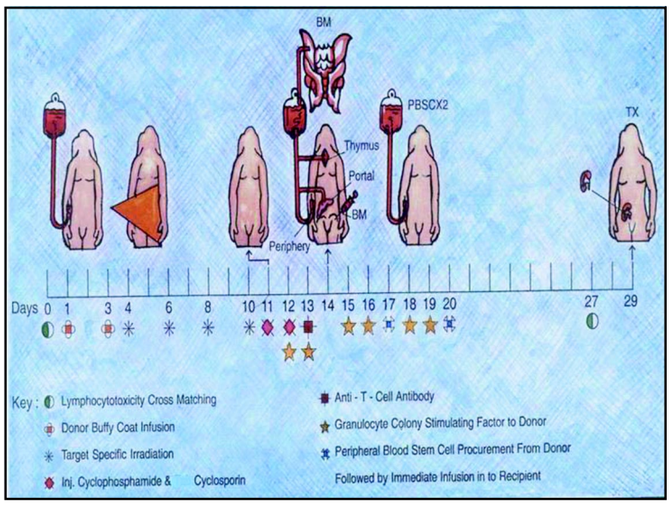

Figure 1 Ahmedabad Tolerance Induction Protocol.

STUDY DESIGN

This was a prospective, open label, randomized control parallel, clinical trial to evaluate the efficacy and safety of tolerance induction protocol associated with MLHC in LRD renal allograft recipients.

Tn was subjected to tolerance induction protocol before undergoing renal transplantation. Cn underwent transplantation directly, after negative lymphocytotoxicity cross-match (LCM).

Patients were screened for the presence of lymphocytotoxic antibodies by LCM serologically before and at the end of tolerance induction protocol which included: donor leucocyte infusion on 1st and 3rd day to stimulate donor-specific T-cell clones; minimal non-myeloablative conditioning which included fractionated low dose target specific irradiation (125 cGy x 4 on alternate days) to abdominal and inguinal lymph nodes, thoracolumbar vertebrae and pelvic bones, from day 4 to 10, to create space for stem cell grafting by deleting resident stem cells, B and T-cells and to delete "cobblestone appearance" colony forming stem cells residing in marrow; administration of cyclophosphamide, 10 mg/kg BW/ day on days 11, 12, as myelosuppressive agent and to create space in thymus, polyclonal anti-T-cell antibody, 1.5 mg/kg BW on day 13, to achieve transient abrogation of alloresistance offered by $CD4^+$/ $CD8^+$ cells by reducing their count below 10 % of normal, and prevent graft versus host disease (GVHD), cyclosporine(CsA), 3 mg/kg BW/day from day 11, to be continued post transplant, as immunosuppressant, to protect chimeric cells and prophylaxis against GVHD and administration of Treosulphan, 1.3 mg/ kg BW on day 11 - 13, as an effective myelosuppressive agent which deletes "cobblestone appearance" colony forming stem cells residing in marrow. Intrathymic donor renal tissue transplantation was done on day 11 to present MHC I/II- rich renal tissue to thymic medulla so that developing double positive ($CD\ 4^+$/ $CD\ 8^+$) thymocytes will register the presence of donor allo-antigens. Donor marrow infusion in to marrow, portal and systemic circulation to achieve grafting was performed on day 14; followed by cytokine stimulated and mobilized peripheral blood stem cells (PBSC) infusion twice on days 17 and 20, to reach the target of 20 x 10^8 cells/ kg BW.

Hematopoietic Stem Cell (HSC) stimulation, mobilization, collection and infusion techniques

LRD of the patients received granulocyte colony stimulating factor, 15 mg /kg BW, subcutaneously for 2 consecutive days followed by bone marrow aspiration from posterior superior iliac crest under general anesthesia. About 400 ml of this aspirated unmodified marrow was infused in to recipient marrow (80 ml), portal (200 ml) and peripheral (120 ml) circulation. This procedure was followed by PBSC collection twice to reach the target of 20 x 10^8 cells/ kg BW of the recipient. Cobe Spectra version 7(Gambro, China) and MCS 3p (Hemonetics, USA) were used for leucopheresis.

Nucleated cell count and CD34 + cell counts were performed after each collection.

HLA and LCM technique

All patients and donors were tested for HLA. LCM was performed using conventional serological techniques (one- Lambda pre-dot trays were used for HLA- A, B, DR typing), using auto cross-match, Dithiothretol and standard cytotoxicity methods with mixed-cell population. T and B lymphocytes were each separately utilized for cross-matching.

Renal transplantation was performed 1 week after the last HSC infusion following negative LCM in Tn and immediately after negative LCM in Cn.

Post transplant follow up

All patients were followed at the same outpatient clinic, at weekly intervals for first 3 months, fortnightly for the next 3 months, monthly intervals for the next 6 months and 3 monthly intervals thereafter. At each visit, renal and liver function status was monitored, complete blood counts were performed and cyclosporine (CsA) levels were measured (using EMIT 2000 CsA specific assay system, Syva Co, Dade Behring, USA). They were monitored at monthly intervals for HIV, HBsAg, HCV, CMV infection status using ELISA technique. Tn recipients were monitored for development of skin rash, fever, jaundice/ gastrointestinal symptoms of GVHD.

Both groups received CsA, 5mg/kg BW/ day, for first month along with prednisolone, 0.5 mg/kg BW/day. Cn received Azathioprine/ mycofenolate mofetil (MMF) as additional third immunosuppressant. CsA was tapered to 3 mg/kg BW/ day in Tn from 2nd month post transplant, and prednisolone was tapered to 0.2 mg/kg BW/ day in both groups from 2nd month post transplant. Cn was continued on the same immunosuppression regime thereafter. CsA dose was adjusted to maintain trough levels of 50-100 ng/ml (recommended trough values: 76-150 ng/ml). Prednisolone was weaned off 6 months post transplant to be discontinued at the end of 9 months post transplant. Thus Tn were kept on low dose (0.5 to 2 mg/kg BW/ day) CsA monotherapy 9 months onwards.

Rejection and treatment

Rejection was diagnosed as per standard modified Banff classification and treated with 3 doses of intravenous methylprednisolone, 500 mg/day [1]. Tacrolimus was used as rescue therapy in steroid resistant rejections.

Chimerism studies

Patients with cross-gender donors from both groups were subjected to peripheral blood chimerism studies before stem cell transplantation (for controls), 1 day before renal transplantation, 100 days after last PBSC infusion and 6 monthly afterwards to look for MLHC.

Peripheral blood (5 ml in heparin) was collected from a subset of patients of both groups. The red cells were lysed using RBC lysis buffer following spinning with phosphate buffered saline at 4-10^0 C for 10 minutes, each time. The lysed cell button was subjected to Cytospin and slides prepared were stained with Geimsa stain. They were then destained and XX/ XY probes (Vysis probes) were applied. They were then studied under microscope for chimerism (The Skyvision Cytogenetics Workstation).

Time Scale (Months)	Cyclosporin (Per Day)	Prednisolone (Per Day)	Azathioprine / mycofenolate mofetil (Per Day)
0 – 1	Tn + Cn (5 mg/Kg BW)	Tn + Cn (0.5 mg/Kg BW)	Cn (2 mg/Kg BW)
2 - 5	Tn + Cn (3 mg/Kg BW)	Tn (0.2 mg/Kg BW) Cn (0.5 mg/Kg BW)	Cn (2 mg/Kg BW)
6 – 9	Tn (2 mg/Kg BW) Cn (3 mg/Kg BW)	Tn (0.1 mg/Kg BW) Cn (0.5 mg/Kg BW)	Cn (2 mg/Kg BW)
10 Onwards	Tn (1 mg/Kg BW) Cn (3 mg/Kg BW)	Cn (0.5 mg/Kg BW)	Cn (2 mg/Kg BW)

Table : 3 Immuosuppression Scheme

RESULTS

Mean total nucleated cell count was 21 x 10^8 cells/kg BW of the recipient with mean $CD34^+$ count 0.8 ± 0.2 %. Serum creatinine (SCr) values were used as indicator of allograft function. A comparative study of SCr values of both groups was undertaken at 3, 6, 9 and 12 months post transplant. It was observed that mean SCr level was significantly better in Tn than Cn. Student's t test (of 2 samples, assuming equal variances) was performed to determine the statistical significance ($p < 0.001$) (figure 2). Over a mean follow up of 372 days of Tn, mean SCr was 1.23 mg /dl with no rejection/ CMV infection/ graft or patient loss. There was transient

rise in donor specific antibodies (mean – 25 %), in all of them which declined to less than 5 % over mean time period of 2 weeks. Three (10 %) patients required 3 plasmapheresis procedures on alternate days to reach the antibody level of 5 %. No adverse effects on allograft function were found in Tn. Fourteen (42.4 %) recipients were biopsied at mean post transplant follow up time of 47.3 days for rise in SCr above 10 % of baseline levels and 13 (92.9 %) biopsies showed acute CsA toxicity in the form of subintimal segmental hyalinosis in small arteries and toxic tubulopathy, one (7.1 %) biopsy showed acute tubular necrosis with acute CsA toxicity. Persistent MLHC was noted in all 21 patients across MHC barriers. Mean MLHC before stem cell transplantation was 0.05 % in 15 who were mother to child transplants, was absent in others, and 100 days after transplantation, it was 0.7 ± 0.3 % (in all of them).

Immunosuppression has been completely withdrawn in 4 patients so far, who were mother to child transplantation recipients (with micro and macro MLHC) with an uneventful mean follow up of 210 days.

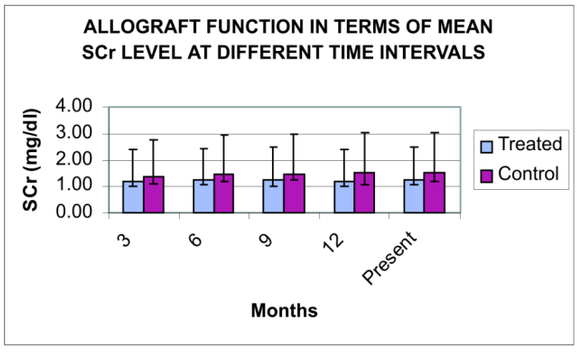

Figure 2

Time Interval (Months)	p Value
3	<0.001
6	<0.001
9	<0.001
12	<0.001
Present	<0.001

Over a mean follow up of 378 days, mean SCr of Cn was 1.52 mg/dl. Twenty two (66.6 %) patients required biopsy at mean follow up of 24.4 days post-transplant, to evaluate the cause of rise in SCr. Acute tubulointerstitial rejection, type IA was observed in 12 (54.6 %) patients, 5 (22.7 %) had acute tubulointerstitial plus acute vascular rejection, type IB +IIA. Acute CsA toxicity was observed in 7 (31.8 %) biopsies. Two patients required re-biopsy at mean follow up of 352.5 days post-transplant, and acute tubulointerstitial rejection on chronic tubulointerstitial plus vascular rejection with chronic transplant glomerulopathy was observed in both of them. CMV infection related graft and patient loss was noted in 1 (3 %) patient at 345 days post-transplant and 1 (3 %) graft loss was observed due to repeated rejection episodes.

DISCUSSION

Over the last five decades transplantation biologists have been trying to define the mechanisms of donor-specific tolerance in solid organ transplantation. Fifty years ago Owen observed that freemartin cattle sharing common placenta exhibited erythrocytic chimerism [2]. Billingham and Medawar were surprised to observe acceptance of grafts between genetically non-identical chimeric twins and that third party grafts were rejected [3, 4]. This link was further confirmed by Billingham, Brent and Medawar where adult spleen and BM derived stem cells were infused in neonatal mice who later on accepted donor skin grafts without immunosuppression. Slavin demonstrated that such tolerance cannot be induced in adult mice without irradiation[5].

We have reported successful implementation of DBMC infusion protocol to induce prope tolerance in clinic [6-9]. We used mega dose allo-HSCs to achieve the benefits of activation induced cell death leading to depletion of potentially rejecting T- cell repertoire utilizing Zinkernagel's concept of MHC restriction [10]. Gorczynski's demonstration of persistent donor specific tolerance in mouse model with portal infusion of allogeneic stem cells instead of conventional systemic route encouraged us to implement this strategy in our patients [11]. We successfully induced prope tolerance by effectively controlling peripheral arm however the central arm (thymus) was yet to be addressed[12]. Posselt's work encouraged us to inoculate the donor renal tissue in to host thymus for exposing donor endothelial cells rich in MHC II expression to the developing thymocytes [13]. We observed the presence of donor-specific regulatory cells immediately after thymic inoculation of donor antigen by performing ELISPOT assay where the T-cell repertoire

was rich in cells secreting Il -10 as compared to lesser population of cells secreting Il -2 (unpublished data). We have successfully validified Medawarian concept of tolerance induction associated with MLHC in human model in 4 (12 %) recipients across MHC barriers.

CONCLUSION

Our data indicates that low level (0.7 to 1%) of MLHC can be achieved across MHC barriers and is associated with induction of donor-specific tolerance in LRD renal transplantation. It can be achieved with safe, low intensity conditioning regime.

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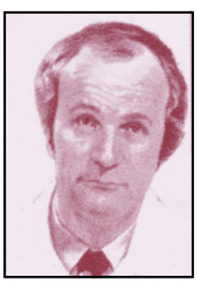

Immunosuppressants and Transplantation

During the 1960s, three major developments, namely the understanding of tissue typing improved methods of obtaining vascular access and better methods of immunosuppression changed the course of clinical transplantation. In 1961, azathioprine quickly became the mainstay of treatment. A more powerful immunosuppressant was not discovered until 1976 when Jean F Borel introduced cyclosporin A. In the early 1980s, antilymphocyte globulin and antithymyocyte globulin became increasingly used.

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