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ALLOGENEIC STEM CELL TRANSPLANTATION, MIXED HEMATOPOIETIC CHIMERISM AND TOLERANCE- FIVE YEARS EXPERIENCE WITH RENAL TRANSPLANTATION

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ABSTRACT

We designed and implemented a clinical protocol to achieve donor-specific, mixed hematopoietic chimerism-associated tolerance in living-related renal allograft recipients using low intensity conditioning and high dose hematopoietic stem cell transplantation (HSCT). This protocol was evolved from zero to low dose conditioning over the period of five years in 481 patients. In our early protocols prope tolerance was achieved with minimum immunosuppressants and there was very low incidence of easily treatable acute rejection episodes and CMV disease. In the fifth protocol we have achieved mixed chimerism-associated tolerance across MHC barriers with no/minimal immunosuppressants and no GVHD in addition to the benefits of earlier protocols.

ABBREVIATIONS

BM	bone marrow	CsA	cyclosporine A
ESRD	end stage renal disease	GVHD	graft versus host disease
HSC	hematopoietic stem cells	HSCT	hematopoietic stem cell transplantation
MP	methylprednisolone	SCr	serum creatinine

INTRODUCTION

We launched our research project of donor-specific tolerance induction in renal transplantation using donor derived hematopoietic stem cells (HSCs) on 15th August, 1998. We would like to define tolerance as renal allograft survival with adequate, stable function without using chronic immunosuppressive support for more than 100 days and normal allograft biopsy. Looking at the prevailing sociological ethos we were well aware that 90% of our patients would be males having mother/ wife (HLA-mismatched) as living-related donors (LRD).

We wanted to validify Medawarian concept of donor-specific tolerance induction by donor specific allogeneic hematopoietic stem cell transplantation (HSCT) in our LRD renal allograft recipients [1, 2]. Our objective was to use non-myeloablative/ minimum conditioning protocols with hematopoietic chimerism

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which would be safe (without graft versus host disease (GVHD)) for patients with end stage renal disease (ESRD) supported on chronic dialysis programme. Institutional Ethics Committee approved the study protocol and consent forms of the trial in accordance with revised declaration of Helsinki.

PATIENTS AND METHODS

Design of protocols (Figures 1 to 4)

All the patients entered the clinical trials following their written consent. Patient demographics are given in table-1.

Proto -col	No.of patients	Gender M:F	Age (yrs)	ETIOLOGY OF ESRD					Donor-Recipient MHC match profile						
				CGN	Obst. Uropa.	DM	ADP KD	Others	0/6	1/6	2/6	3/6	4/6	5/6	6/6
A	27	22:5	37 (10-61)	10	0	3	2	12	9	7	3	4	3	1	0
B	234	208:26	33 (10-69)	97	3	16	7	111	35	49	49	87	10	1	3
C	70	60:10	34 (12-58)	33	1	6	1	29	9	14	16	24	7	0	0
D	100	87:13	34 (11-64)	42	3	5	3	47	10	24	27	28	6	5	0
E	50	44:6	34 (15-60)	8	3	5	1	33	9	12	6	19	2	2	0

Table 1

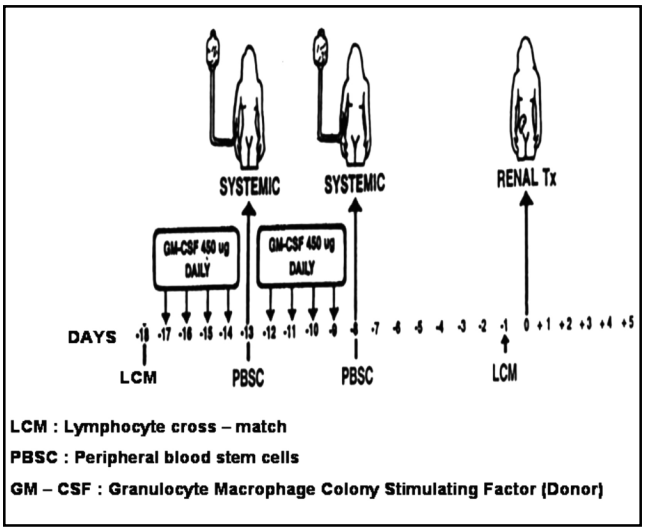

Figure 1 Protocol A

The first protocol (A) was implemented in 27 patients transplanted from September 1998 to December, 1998. We aimed at infusion of donor derived HSCs in renal allograft recipients pre-transplantation without any conditioning regimen. We aimed at infusion of 10 x 10^8 unmodified peripheral blood stem cells / kg BW.

In second protocol (B) for 234 patients transplanted from October 1998 to September 2002, we modified the previous

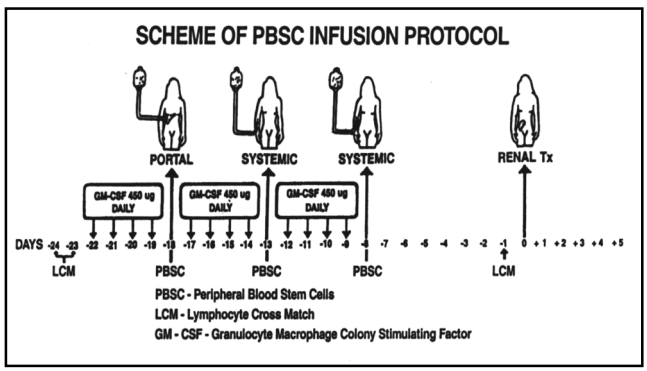

Figure 2 Protocol B

protocol by increasing the HSC dose from 10 to 15 x 10^8 cells and included portal infusion in this protocol.

In third protocol (C) for 70 patients transplanted from end of January 1999 to May 2002, we further modified our protocol by adding inoculation of donor bone marrow (BM) derived cells in to recipient thymus to the protocol B.

In fourth protocol (D) for 100 patients transplanted from March 2002 to October 2003, we modified protocol C by intrathymic transplantation of donor antigen (kidney tissue) instead of BM derived cells.

The benefits of protocols were recognized in the form of prope tolerance and overall better allograft function with lower

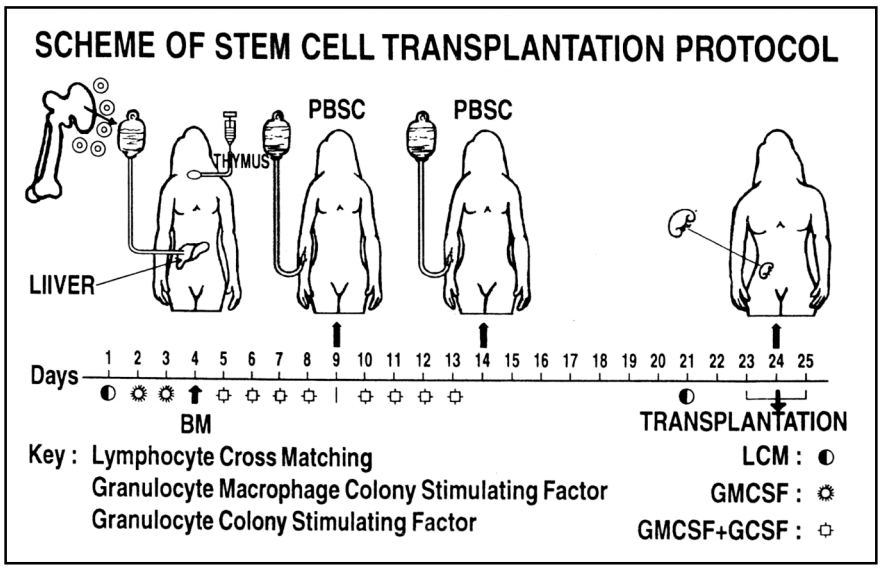

Figure 3 Protocol C

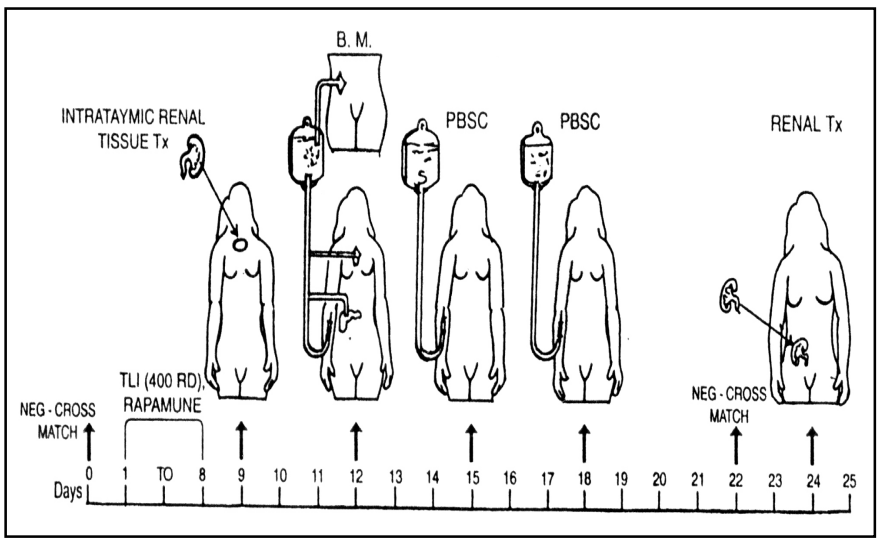

Figure 4 Protocol D

incidence of rejections and infections, with each modification. However we were not able to reliably reproduce demonstrable mixed hematopoietic chimerism with robust permanent tolerance in all patients. So, we decided to modify protocol D.

The fifth modified protocol E was implemented October 2003 onwards. This protocol included 2 donor specific transfusions (DSTs) and low intensity conditioning. The first step was to infuse 2 DSTs (which included only separated buffy coats and remaining blood was returned to the donor to prevent plasma and RBC loss) at intervals of 2 days to stimulate proliferation of donor-specific alloreactive T-cell clones followed by series of steps to delete proliferated and proliferating T-cell clones. This was believed to create "space" in thymus, BM and lymph nodes. The conditioning regime was initiated with fractionated (4 doses on alternate days) low dose (400 cGy) target-specific (abdominal and inguinal lymph nodes, BM of vertebral bodies and part of hip bones) irradiation. Then we administered polyclonal anti-T-cell antibody (rabbit), 1.5 mg/kg BW along with cyclophosphamide, 20 mg/kg BW. Cyclophosphamide was used to create "space" in thymus by deletion of allo-resistive host antigen presenting cells in thymus. Cyclosporine, 3 mg/kg BW was added to protect chimerism, create space in thymus and prevent GVHD. Within 24 hours of achieving deletional target of host CD4+/ CD8+ count to less than 10 %, unmodified donor BM stem cells (400 ml) were infused (80 ml in to sternal BM, 200 ml in to portal circulation and 120 ml in periphery). It was supplemented by infusion of 2 cytokine stimulated peripherally mobilized stem cells at intervals of 2 to 3 days (Cobe Spectra 7, China, and Hemonetics-MCS3p, USA). We aimed at infusing at least 20 x 10^8 cells/ kg BW. Renal transplantation was performed about 1 week after the final HSC infusion following negative lymphocytotoxicity cross matching (LCM). All patients who developed donor-specific cytotoxic antibodies were treated with cyclosporine and cyclophosphamide. In case of high positivity intravenous gamma globulins and plasmapheresis were also used. Transplantation was followed as soon as negativity was achieved. In this fifth protocol (E) we have transplanted 50 patients from October 2003 to May 2004.

HSC mobilization, collection and infusion techniques

LRD of patients were administered GM-CSF, 10 microgram/ kg BW in 2 divided doses subcutaneously for 4 days for BM stimulation, followed by leucopheresis on 5th day. Unmodified

PBSCs collected by apheresis technique were immediately infused in to periphery in protocol A.

The first collection (PBSC in protocol B and BM in all other protocols) was administered in portal circulation through omental vein, under general anesthesia in all other protocols. PBSC was repeated once/ twice to meet the target of 15-20 x 10^{8+} cells/ kg BW of recipient.

In protocols C and E, we added concentrated BM inoculation in to recipient thymus as the first step. A 4 cm long incision was made in to the right second intercostal space under general anesthesia. After cutting all the muscles, mediastinal fascia was opened and thymus was identified in the retrosternal space.

We collected first 5 ml of BM from donor posterior superior iliac crst before collecting BM for portal, intra-marrow and peripheral infusion. This BM was centrifuged for 10 minutes at 1500 RPM and supernatant plasma was removed. Concentrated BM inoculum of 1.5 ml was then injected in to thymus with 20 guaze needle, hemostasis checked and wound closed. Then 200 ml of BM was injected portally, 80 ml in to sternal marrow and 120 ml in to periphery.

In protocol D recipients received donor-renal tissue with mean weight of 600 micrograms and mean total glomeruli, 12, procured with standard kidney biopsy procedure under local anesthesia and inoculated into recipient thymus after mincing.

HLA typing and cross-matching by LCM

HLA typing and LCM were performed at the beginning and end of tolerance induction protocols using conventional serological techniques (one- Lambda pre-dot trays were used for HLA- A, B, DR typing), using auto cross-match, DTT and standard cytotoxicity methods with mixed-cell population. T and B lymphocytes were each separately utilized for cross-matching and donor-specific positivity was found with mixed-cell population.

Transplantation and follow-up

Transplantation was performed following negative LCM, one week after the last HSC infusion. All patients of all protocols were followed up at the same out-patient clinic at weekly intervals for the first three months, every two weeks for the next 3 months, at monthly intervals for the first year and every three months subsequently. Each visit included monitoring of their liver and renal function status, complete blood counts and CsA levels. They were monitored at monthly intervals for HIV, HBsAg, HCV and CMV (IgG and IgM antibody) infection status in the first year and at three monthly intervals thereafter (using ELISA technique).

Recipient immunosuppression

CsA was the principal immunosuppressant, doses were adjusted with an intention to maintain trough blood levels of 50-176 ng/ml. CsA levels were monitored by employing EMIT 2000 CsA specitic assay. Prednisolone, 0.4 mg/ kg BW/ day was also used with the intent to discontinue 6 months post-transplantation.

Diagnosis of rejection and its treatment

Allograft biopsy was performed in all recipients for rise in s. creatinine of more than 10 % above baseline. Modified Banff criteria were used for diagnosis of graft pathology including rejection [3]. Rejection was treated with intravenous methylprednisolone, 250 mg/kg BW/day for 3 consecutive days.

Chimerism studies

A subset of patients with donors of opposite gender were subjected to chimerism studies using peripheral blood by fluorescent in-situ hybridization technique (Carl Zeiss microstation using Vysis probes for X/Y probes) at random time intervals.

RESULTS

The profile results of all protocols are mentioned in table 2.

Protocol A

Out of 27 patients with mean follow-up time of 1838.7 days, 18 (66.7 %) patients have adequately functioning grafts. All of them are on Prednisolone, 0.2 mg/kg BW and CsA, 2 mg/kg BW/day or Azathioprine, 2 mg/kg BW/ day. Acute rejection which responded to anti-rejection treatment was observed in 2 (7.4 %) patients, who subsequently had CMV disease. Chronic rejection was observed in 4 (14.8 %) patients who eventually lost their allografts. We lost 5 (18.5 %) patients with functioning grafts due to serious infections and cardiovascular events.

Protocol B

Out of 234 patients with mean follow-up time of 1514.2 days, 195 (83.3 %) patients have adequately functioning grafts. All

Protocol	PBSC	PBSC+ Portal	PBSC+HSC in Thymus	PBSC+Portal, Thymic Tissue Inoculation	T – Protocol
Period	1/9/98 to13/2/01	2/10/98to 5/9/02	27/1/99 to13/5/02	13/3/02 to 5/2/04	16/10/03 onwards
Follow Up	1838.7	1514.2	1002.2	457.1	101.7
Pts	27	234	70	100	50
Graft Function %	18 (66.7%)	195 (83.3 %)	64 (91.4%)	96 (96%)	50 (100%)
CR (%)	4 (14.8%)	27 (11.5 %)	2 (2.9%)	0	0
Death (%)	5 (18.5%)	9 (3.9%)	4 (5.7%)	4 (4%)	0
	2 – Cardiac	5 – Infection	4 – Infection		
	3 – Infection	4 – Non Infection			
Recurrent	0	3 (1.3%)	0	0	0
Disease (%)					
Acute rejection (%)	2 (7.4 %)	14 (5.9%)	2 (2.9%)	0	0
CMV disease	2 (7.4 %)	11 (4.7%)	3 (4.3%)	0	0
Pt. Loss	5	9 (3.8%)	4 (5.7%)	4 (4%)	0
Graft Loss	4 (14.8 %)	27 (11.5%)	2 (2.9%)	0	0

Table 2 Profile of Results of Protocol

of them are on Prednisolone, 0.2 mg/kg BW and CsA, 2 mg/kg BW/day or Azathioprine, 2 mg/kg BW/ day. Acute rejection which responded to anti-rejection treatment was observed in 14(5.9 %) patients and CMV disease was recorded in 11 (4.7 %) patients. Chronic rejection was observed in 27 (11.5 %) patients who eventually lost their allografts. We lost 9 (3.8 %) patients with functioning grafts due to serious infections and cardiovascular events.

Protocol C

Out of 70 patients with mean follow-up time of 1002.2 days, 64 (91.4 %) patients have adequately functioning grafts. All of them are on Prednisolone, 0.2 mg/kg BW and CsA, 2 mg/kg BW/day or Azathioprine, 2 mg/kg BW/ day. Acute rejection which responded to anti-rejection treatment was observed in 2 (2.9 %) patients; 3 patients had CMV disease. Chronic rejection was observed in 2 (2.9 %) patients who eventually lost their allografts. We lost 4 patients with functioning grafts due to chronic rejections precipitated by CsA toxicity.

Protocol D

Out of 100 patients with mean follow-up time of 457.1 days, 96 (96 %) patients have adequately functioning grafts. All of them are on Prednisolone, 0.2 mg/kg BW and CsA, 2 mg/kg BW/day or Azathioprine, 2 mg/kg BW/ day. There was no acute or chronic rejection/ CMV disease. We lost 4 (4 %) patients with functioning grafts due to infections and cardiovascular events.

Protocol E

Out of 50 patients with mean follow-up time of 106.6 days, all patients have adequately functioning grafts without any rejection episode or infections. Most of them are on Prednisolone, 0.2 mg/kg BW and CsA, 2 mg/kg BW/day or Azathioprine, 2 mg/kg BW/ day.

Eight patients of protocol E, and occasional patients from all protocols evaluated for chimerism have demonstrable lymphohematopoietic chimerism (0.09$\pm$ 0.22 in all patients, versus 0.38 $\pm$ 0.46 in protocol E).

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 [4]. Billingham and Medawar were surprised to observe acceptance of grafts between genetically non-identical chimeric twins and that third party grafts were rejected [1, 2]. 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 [6-8]. We were desirous of achieving the benefits of activation induced cell death leading to depletion of potentially rejecting T- cell repertoire utilizing Zinkernagel's concept of MHC restriction [9]. Hence we decided to use megadose allo-HSCs.

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 [10]. Donor stem cells when infused in portal circulation get trapped in hepatic microcirculation and initiate a series of events leading to peripheral tolerance.

All patients were not doing well in spite of applying these strategies. We achieved prope tolerance in earlier protocols (A, B) however we were not able to prevent smouldering rejections. We were able to create an effective peripheral tolerance. However we had not addressed to the central arm of tolerance. Remuzzi inoculated donor alloantigens in to the recipient thymus in rodent model to create classical central tolerance [11]. We have implemented this principle by inoculating donor derived BM in to thymus in protocol C.

We were not able to prevent chronic rejection although the incidence and intensity of acute rejection episodes became significantly less, easily and effectively treatable; and the allograft function remained stable and better with minimum incidence of viral/ bacterial infections, but the goal of achieving drug-free allograft survival without chronic rejection eluded us. This brought us to a point of improvising upon our protocol by replacing donor derived stem cells with donor derived kidney tissue for thymic inoculation. This was implemented after reviewing Posselt's work [12]. We aimed at exposing donor endothelial cells rich in MHC II expression to the developing thymocytes. 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). All recipients of protocol D attained very good, stable allograft function.

Waldman's experimental work with mouse model demonstrated that maximum alloresistance to the grafting of HSCs from a mismatched donor comes from host CD4 + T-cell population [13]. He further demonstrated that grafting in this situation was achievable when CD 4+ count was decreased to less than 10% with anti-T-cell antibodies (optimal effect). The most suitable time for successful grafting was within 3 days of optimal effect. Hence we used polyclonal anti-T-cell antibodies (rabbit) to achieve target of optimum deletion of host CD4-CD8 T-cell population. BMT was performed immediately after achieving this target. This strategy is effective for peripheral deletion only. Host thymocytes however remain resistant to this process. We therefore used Cyclophosphamide and CsA to make deletional space in thymus.

Slavin et al have reported a strategy of tolerance induction in which they have used low dose lymphoid irradiation, DST to stimulate donor-specific alloreactive T-cells and cyclophosphamide to delete these stimulated clones. Second and final T-cell depleted HSCT was required to establish tolerance [5]. Exner et al have demonstrated that stable, multi-lineage chimerism could be achieved with anti-CD 4/ anti-CD8 antibodies, 300 cGy total body irradiation and cyclophosphamide, 50 mg/kg BW [14]. Chimerism with 1% donor cells was found to be as tolerant as 98% chimerism.

So far, we achieved early, stable and adequate allograft function with minimum immunosuppression, but we were not able to demonstrate presence of mixed chimerism in all patients. So we decided to be aggressive in our approach of conditioning and added DSTs, low dose intensity conditioning regime, polyclonal anti-T –cell antibodies, cyclosphophamide and cyclosporine to delete the peripheral proliferating and proliferated donor specific alloreactive T-cell clones and also to create "space" in thymus, BM and lymph nodes. Thymic