transplantation
india

TRANSPLANTATION TOLERANCE WITH NATURAL SUPPRESSOR CELL CHIMERISM IN LIVING RELATED DONOR RENAL ALLOGRAFT RECIPIENTS- AHMEDABAD EXPERIENCE

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ABBREVIATIONS

BM:	Bone marrow	Cn:	Controls
CsA:	Cyclosporin	ESRD:	End stage renal disease
G-CSF:	Granulocyte colony stimulating factor	GVHD:	Graft versus host disease
HSC:	Hematopoietic stem cells	HSCT:	Hematopoietic stem cell transplantation
LCM:	Lymphocytotoxicity cross match	LRD:	Living related donor
NSC:	Natural suppressor cells	NSCC:	Natural suppressor cell chimerism
Renal Tx:	Renal Transplantation	SCr:	Serum creatinine
Tn:	Treated		

KEY WORDS

natural suppressor cell chimerism, renal transplantation, tolerance

ABSTRACT

Aims

We designed prospective randomized-control trial to evaluate efficacy of tolerance induction protocol in living-related donor(LRD) renal transplantation(RTx) using hematopoietic stem cell transplantation (HSCT), and low intensity conditioning-induced natural suppressor cell chimerism (NSCC) measured by flow cytometry(FACScan).

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Methods

Patients divided in 2 equal groups of 200 each were enrolled for LRDRTx: treated(Tn) and control(Cn), balanced in demographics, HLA match profile.

Tn received HSCT, 20x10^{8}cells/kg BW, conditioning with low-dose irradiation, anti-T-cell antibodies, Treosulfan, cyclophosphamide, Cyclosporin(CsA). Donor BM was infused in thymus, BM, portal, peripheral circulation, supplemented by peripherally mobilized HSCs. RTx was performed after negative lymphocytotoxicity cross-match. NSCC was monitored 3 monthly postTx by FACScan, confirmed by fluorescent-in-situ hybridization(FISH) in cross-gender Tx.

Cn received 3 drug immunosuppression. Tn received Prednisolone, 0.2 mg/kg/day +CsA, 3 ±0.5mg/kg/day. Trough CsA levels were maintained around 100ng/ml. Immunosuppression was discontinued 1 year postTx after cytoanalysis in Tn.

Tolerance was defined as stable graft function for >100 days after drug withdrawal+ unremarkable allograft biopsy.

Results

Over mean follow-up of 34.7 months, Tn had stable graft function with mean serum creatinine(SCr), 1.3 mg%, low rejection, no patient/graft loss. Mean peripheral donor CD3^{+dim}: 4%, CD34$^+$/33$^+$:0.1 % was noted in Tn, FISH revealed mean 0.08% donor cells in periphery, 25 patients achieved tolerance; 171 are on drug withdrawal.

Over mean follow-up, 35.1 months, mean SCr of Cn was 2.7 mg%, 65.5% had rejection, 36% grafts and 24% patients were lost. Mean donor NSCC was significantly less in Cn.

Conclusion

We achieved tolerance across MHC barriers in LRDRTx with peripheral donor NSCC.

INTRODUCTION

The understanding of mechanisms of tolerance in experimental neonatal mouse model and its resurrection in clinic required synthesis and application of several complimentary themes which have been pursued for the last fifty years by different groups of investigators.

This has been the challenge for transplantation biology and has therefore attracted attention of intellectuals in the field. Donor specific bone marrow(BM) infusions have been observed to be effective in varying degrees in every transplanted tissue/organs of various species including mouse, dog and non-human primates.

We developed a comprehensive method with an aim to induce allogeneic hematopoietic stem cell (HSC) grafting resulting in mixed hematopoietic chimerism associated tolerance across MHC barriers in clinic which would be safe and efficacious. There were two principal themes of this protocol: donor specific alloreactive lymphocyte depletion followed by establishment of donor specific chimerism. Efficacy of this protocol depended upon stimulation-depletion of peripheral T-B cell repertoire and establishment of permanent donor specific chimerism particularly in thymus.

METHODS

Study Design

We carried out a prospective, randomized, open labeled, simultaneous control clinical trial in our living-related donor (LRD) renal allograft recipients from September 1998 to April 2005 at the Institute of Transplantation Sciences and Institute of Kidney Diseases and Research Centre, Ahmedabad, India, to evaluate the efficacy and safety of tolerance induction protocol in inducing mixed hematopoietic chimerism associated donor-specific tolerance. We also devised a cyto-analytical method to identify and correlate with clinical tolerance, and sequentially monitor a subset of donor natural suppressor cell chimerism (NSCC) in BM and periphery of treated group(Tn) and compared with controls (Cn). The trial was conducted in accordance with revised Declaration of Helsinki. Institutional Ethics Committee approved the study protocol and consent forms of clinical trial.

Selection of patients

Four hundred consecutive patients admitted with end stage renal disease (ESRD) for transplantation were approached with intent to treat and their informed consent was obtained. They were distributed in two equal groups of 200 patients each: Tn and Cn. Patient demographics and baseline characteristics

are summarized in tables 1 and 2. The 3 most common etiologies of ESRD were chronic glomerulonephritis, obstructive uropathy and reflux nephropathy. Patients with hepatitis B/C virus infections were excluded from this study. All patients were followed-up at the same out-patient clinic at weekly intervals for the first 3 months, fortnightly for the next 3 months, monthly intervals till 1 year post-transplant and 3 monthly thereafter. In every visit their renal, liver functions and complete blood counts were monitored, and cyclosporine A (CsA) levels were measured using EMIT 2000 assay (Syva Co., Dade Behring, USA, with trough levels of 50 to176 ng/mL). HIV, HBsAg, HCV and CMV (IgG/ IgM antibodies) infection status was monitored using ELISA technique at monthly intervals in the first 6 months and every 3 months Thereater. Recipients of Tn were monitored for development of skin rashes, fever and gastrointestinal symptoms of graft versus host disease (GvHD) for 1 year posttransplant. Their liver and BM functions were also monitored at monthly intervals during the same period.

Patients	Tn (n=200)	Cn (n=200)
Gender – M:F	175:25	174:26
Mean Age (range) (Yrs)	31 (10 -51)	34 (8 -55)
Average 3rd party infusions	14	16
CMV Seropositivity		
Patients	33 (94.2 %)	32 (91.4 %)
Donors	32 (91.4 %)	31 (88.5 %)
Etiology of ESRD		
Chronic Glomerulonephritis	78 (39 %)	81 (40.5 %)
Obstructive Uropathy	17 (8.5 %)	21 (10.5 %)
Reflux Nephropathy	14 (7 %)	17 (8.5 %)
Hypertensive Nephropathy / Chronic interstitial nephritis (each)	13 (6.5 %)	09 (4.5 %)
Chronic Pyelonephritis	10 (5 %)	09 (4.5 %)
Diabetic Nephropathy	06 (3 %)	07 (3.5 %)
MPGN	08 (4 %)	09 (4.5 %)
FSGS	09 (4.5 %)	08 (4 %)
Primary IgA nephropathy	06 (3 %)	07 (3.5 %)
Alport Syndrome	08 (4 %)	07 (3.5 %)
Miscellaneous	18 (9 %)	16 (8 %)

Table1 Patient Demographics

n=200	0/6	1/6	2/6	3/6	4/6	5/6	6/6
Tn	28	32	28	100	10	2	0
Cn	33	34	33	97	3	0	0

Table 2 Donor -Recipient HLA match profile

NSCC using flow cytometry and fluorescent in-situ hybridization(FISH) studies

NSCC was studied using FACScan (Becton Dickinson, CA, U.S.A.) and FISH using Nikon micro-station (Japan). In flow cytometry, we analyzed $CD3^{+}CD4^{-}CD8^{-}$, $CD3^{dim}CD4^{-}CD8^{-}$, $CD34^{+}CD45^{weak}CD33^{weak}$ cell lines every 3 months posttransplant in peripheral blood and at 6 monthly intervals in BM samples collected from posterior superior iliac crest of all recipients. We used CD3 mAb(PerCP conjugated), CD4 mAb (fluorescein isothiocynate (FITC) conjugated) and CD8 mAb (phycoerythrin(PE) conjugated). For CD 33/ 34/45 cell line analysis, we used CD33 mAb (PE conjugated), CD34 mAb (FITC conjugated) and CD45 m Ab (Per CP conjugated). The monoclonal antibodies were purchased from B.D. Biosciences, CA, U.S.A.

Recipient peripheral blood and BM samples were collected in EDTA. Single cell suspensions from these were prepared and stained for 30 minutes at 4^{0}C using specific markers as mentioned above. Cells were analyzed on FACScan. Unstained blood samples were used as negative controls.

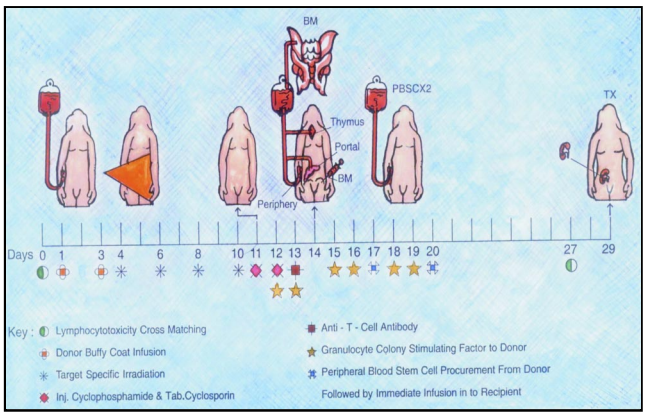

Figure 1 Ahmedabad Tolerance Induction Protocol

FISH was used as a confirmatory test in gender mismatched transplantation. We looked for donor cells which were of

opposite gender in the same samples collected above using Vysis (XX/XY) probes (IL, U.S.A.).

Step 1: Donor leucocyte infusions were carried out twice, on days 1 and 3.

Step 2: Target specific low dose fractionated irradiation (400 cGy x alternate days) to sub-diaphragmatic lymph nodes, spleen, vertebral bodies and part of pelvic bones on days 4, 6, 8, 10.

Step 3: CsA, 3 mg/kg BW/ day, was started on day 11 after irradiation and continued posttransplant. Simultaneous non-myeloablative low intensity conditioning was done with cyclophosphamide, 10 mg/kg BW/day, on days 11 and 13; polyclonal anti-rabbit T-cell antibody, 1.5 mg/Kg BW and Treosulfan, 15 ng / Kg BW each, in a single dose on day 13.

Step 4: High dose HSC administration in unmodified form with target of $\geq 20 \times 10^8$ nucleated cells / kg BW was performed on day 14. This was administered in thymus, marrow, portal and peripheral circulation.

HSC mobilization, collection, infusion and inoculation techniques

Donors received Granulocyte-Colony Stimulating Factor (G-CSF), 10 *u*g /kg BW /day subcutaneously on days 12, 13. BM aspiration (400 ml) was performed under sedation and local anesthesia from their posterior superior iliac crest after cytokine stimulation. We inoculated approximately 1.5 ml of concentrated donor BM in recipient thymus. Four cm long incision was made in to the right second intercostal space of recipient under general anesthesia. After cutting all the muscles, mediastinal fascia was opened and thymus was identified in the retrosternal space. Then 1.5 ml of concentrated marrow was inoculated with 20 gauze needle. Hemostasis was checked and wound closed. Mini laparatomy was performed simultaneously for portal infusion. Omental vein was identified and canulated. We infused 200 ml of the collected BM through this canula, which was removed after infusion, hemostasis was checked and the wound closed. We infused 40 ml each, of the above marrow in to bilateral anterior superior iliac crests. The remaining 200 ml was infused in peripheral circulation.

Step 5: Subsequently G-CSF, 10 *u*g/ kg BW/ day, was continued to stimulate and mobilize HSCs. Donors were then subjected to leucophoresis on days 17 and 20, on stem cell separator (Hemonetics, MCS 3p, USA and Cobe Spectra version 7, Gambro, China) and peripheral blood stem cells (PBSC) were collected and immediately infused in unmodified form in the periphery of recipients. Each stem cell inoculum was subjected to cell count for total nucleated, differential and CD34+ cell counts.

Tolerance induction protocol was completed in 21 days and patients underwent kidney transplantation after negative lymphocytotoxicity cross-matching (LCM) performed about 1 week after the last PBSC infusion.

HLA typing and cross-matching by LCM

HLA typing and LCM were performed at the beginning and end of tolerance induction protocol 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.

Immunosuppression (mg/Kg BW/Day)		Time scale in months			
		0 - 1	2 –5	6 - 9	10 Onwards
CsA-	Tn	5	3	2 ± 1	1 ± 0.5
	Cn	7	7	4 ± 2	4 ± 2
Prednisolone	-Tn	0.5	0.2	0.1	-
	Cn	0.5	0.5	0.5	0.5
Aza /	-Tn	-	-	-	-
MMF (2 gm/day)	Cn	2	2	2	2

Table 3 Recipient Immunosuppression

Desensitization protocol for sensitized host following stem cell infusion

Splenic irradiation (300 cGy in divided doses), plasmapheresis (one to three procedures) and administration of MMF, 2 gm / day in two divided doses was employed in the event of occurrence of donor-specific cytotoxic allo-antibodies. Renal transplantation was performed (in mean time period of 9 days) as soon as LCM negativity was achieved.

CsA was the principal immunosuppressant for both groups. The doses were adjusted with an intention to maintain trough blood levels of 120 ± 20 ng/ ml. Prednisolone, 0.5 mg/kg BW /day was administered for the first 3 months post-

transplantation followed by 0.2 mg/kg BW/ day subsequently. Azathioprine, 2 mg/kg BW/ day was added as a third drug following acute rejection (AR) episodes. Azathioprine was replaced by mycofenolate mofetil (MMF) whenever toxicity was observed. Patients were switched over from CsA to Azathioprine at the end of 1 year posttransplant in Tn. Cn required Azathioprine/ MMF as a third immunosuppressant.

Immunosuppression withdrawal in Tn

At the end of 1 year posttransplantation, if peripheral $CD3^{dim}$ levels were more than 3.6 % persistently at 15 months posttransplant, immunosuppression was withdrawn over 3 months. Thus patients were off all immunosuppression at 18 months posttransplant. Graft biopsy was performed after 100 days of complete drug withdrawl.

Diagnosis of rejection and its treatment

All recipients were biopsied whenever clinically suspected for rejection. Rejection was diagnosed according to the modified Banff criteria and treated with intravenous methylprednisolone (MP), 250 mg/day for three consecutive days [1]. FK 506 (Tacrolimus) was used as rescue therapy in MP-resistant rejections.

Parameters	Tn (n=200)	Cn (n=200)
Mean Follow up (Mths)	34.7 (7 – 80)	35.1 (7 - 80)
Rejection	Borderline ATIR-1% $C4d^{-}$ AVR I - 1%	131 (65.5 %)
Graft Loss	0	72 (36 %)
Patient Loss	0	48 (24 %)
Mean S.Cr (mg %)	1.33 (0.7 – 1.95)	2.76 (0.9 -5.6)

Table 4 Results

Tn with an average follow-up of 34.7 months (range: 7-80 months) showed significantly better graft function and had very low incidence of acute rejection (AR) episodes. There was acute borderline tubulo-interstitial rejection (ATIR) in 2 (1 %) patients and $C4d^{-}$ acute vascular rejection (AVR), type IIA, in 2 (1 %) patients. No CMV infection was seen. There was no graft/ patient loss in Tn.

Tolerance

We observed 3 kinds of tolerance in Tn. Robust tolerance (stable graft function for more than 100 days without any immunosuppression) was achieved in 25 (12.5 %) patients, prope tolerance (early, adequate, stable graft function with no rejection episodes with minimum immunosuppression) was achieved in 171(85.5 %) patients. Metastable tolerance (adequate graft function on minimum immunosuppression with single episode of steroid responsive acute rejection episode) was observed in 4 (2 %) patients. All 25 patients with robust tolerance had unremarkable graft biopsies.

One hundred and twenty one (60.5 %) patients were successfully switched from CsA to MMF/ Azathioprine 1 year post-transplantation and Prednisolone was also discontinued in all of them. The remaining 50 patients have not completed 1 year of transplantation and are on low dose CsA and Prednisolone.

In Cn with an average follow-up of 35.1 months (range:7-80 months) 6 (3%) patients had AVR, type IIA, 5 (2.5 %) AVR IIB, 48 (24 %) had ATIR, type IB, 13 (6.5 %) had ATIR type IB+ AVR type IIA, 19 (9.5 %) had ATIR type 1A +AVR type IIB and 50 (25 %) patients had ATIR, type IB +AVR type II A with chronic transplant glomerulopathy, arteriopathy as well as rejection which ultimately led to graft loss. Out of 72 (36 %) patients who lost their grafts, 22 did not recover with rescue therapy and lost their grafts to progressive function loss, uremia and multiple infections. We lost 48 (24 %) patients. The remaining patients have an average SCr of 2.76 mg % (range: 0.9-5.6 mg %). CMV infection was noted in 85 (42.5 %) patients out of whom 63 recovered after adequate treatment with Gancyclovir. A comparative study of SCr values of all the groups was undertaken at 1, 3, 6, 9 and 12 months respectively using student's paired t test and Tn had significantly better graft function ($p<0.001$) (fig 2).

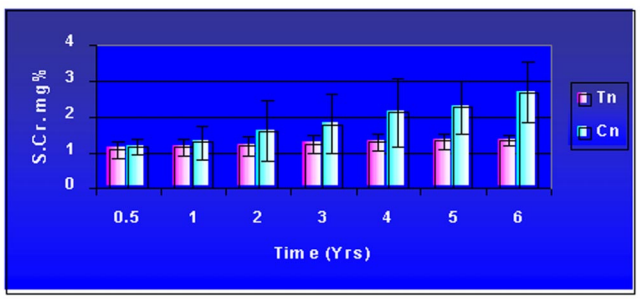

Figure 2 Graft function in terms of S. Creatinine of Treated group (Tn) vs Controls (Cn)

None of the patients from Tn had GvHD. Transient rise of donor specific cytotoxic allo-antibodies was noted in all

patients of Tn at the end of tolerance induction protocol. Desensitization protocol was effectively used in 5 (2.5 %) patients and the remaining patients returned to transplantable range of donor-specific cytotoxic antibody levels within 2 weeks. FK 506 rescue therapy was effective in 47 (68.1 %) patients out of 69 on whom it was used.

PostTx	Tn		Cn	
-	$CD3^{dim}$ Periphery /BM	CD33/34 Periphery /BM	$CD3^{dim}$ Periphery /BM	CD33/34 Periphery /BM
6 months	3.6 ± 1.5/ 5.85± 1.5	0.09 ± 0.01/ 0.33 ± 0.1	2.05± 1/ 3.6 ± 1.1	0.03 ± 0.01/ 0.21 ± 0.01
1 yr	3.8 ± 1.4/ 5.6 ± 1.4	0.09 ± 0.01/ 0.33 ± 0.1	2.9 ± 0.9/ 2.5 ± 1	0.03 ± 0.01/ 0.18 ± 0.01
5 yrs	3.4 ± 1.1/ 5.9 ± 1.1	0.06 ± 0.01/ 0.71 ± 0.01	2.63 ± 1/ 1.92 ± 0.9	0.04 ± 0.01/ 0.19 ± 0.01

Table 5 Natural Suppressor Cell Chimerism Analysis

The side effects of G-CSF injection to donors were malaise (90%), mild pyrexia (89 %) and occasional skin rashes which responded to anti-pyretic and anti-histaminic agents. None of the donors had serious or life-threatening reactions. Portal, thymic and intra-marrow inoculation were uneventful.

We observed two cell lineages; $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak}, stabilizing from 3.6 ± 1.5 % and 0.09 ± 0.01 % respectively at 6 months post-transplantation to 3.8 ± 1.4 % and 0.11 ± 0.01 % in peripheral blood 1 year post-transplantation in Tn. $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak} in BM were 5.85 ± 1.5 % and 0.33 ± 0.1 % respectively at 6 months post-transplantation and 5.6 ± 1.4 % and 0.58 ± 0.1 % at 1 year post-transplantation. We have found stable persistent levels of peripheral chimerism for more than 5 years in a subset of our patients in whom $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak} levels were 3.4 ± 1.1 % and 0.06 ± 0.01 % respectively; $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak} in BM were 5.9 ± 1.1 % and 0.71 ± 0.01 % respectively in this group of patients.

In Cn, peripheral $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak} were 2.05 ± 1 % and 0.03 ± 0.01 % respectively at 6 months post-transplantation, 1.05 ± 0.9 % and 0.04 ± 0.01 % at 1 year post-transplantation. In BM, $CD3^{dim}CD4^{-}CD8^{-}$ and $CD34^{+}CD33^{weak}$CD 45^{weak} were 3.6 ± 1.1 % and 0.06 ± 0.01 % respectively at 6 months post-transplantation, 2.9 ± 0.9 % and 0.03 ± 0.01 % at 1 year post-transplantation.

Peripheral $CD3^{dim}$ levels in robust tolerance were >3.67 %, in prope tolerance were 3.6 % and metastable tolerance were 3.5 %. The other cell levels were not significantly different in all groups (figures 3-6).

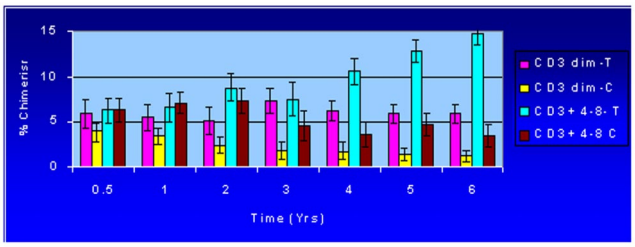

Figure 3 Peripheral Blood Chimerism Studied in Terms of $CD3^{dim}$ Levels in Treated (Tn) vs Controls (Cn)

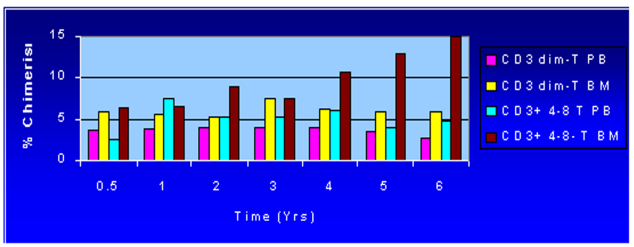

Figure 4 Bone Marrow Chimerism Studied in Terms of $CD3^{dim}$ Levels in Treated (Tn) vs Controls (Cn)

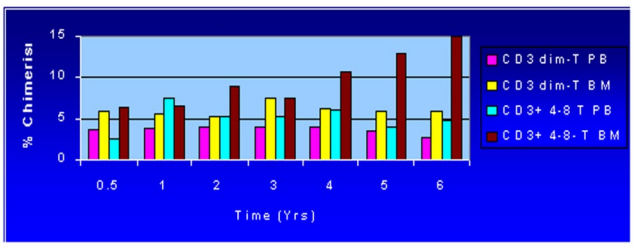

Figure 5 Back Home: Migration of NS cells From Periphery to BM

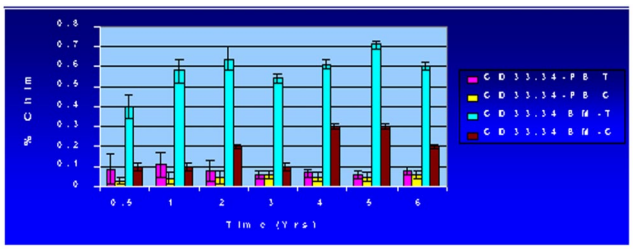

Figure 6 CD 33/34 Cell Levels in Treated (Tn) Group vs Controls (Cn) in Periphery and Bone Marrow

FISH studies revealed 0.6 ± 0.2 % donor HSC in BM and 0.05 ± 0.02 % in periphery in Tn and in Cn there was no donor HSC in either BM or periphery.

DISCUSSION

Over the last five decades transplantation biologists have been trying to define the mechanisms of donor-specific tolerance in solid organ transplantation. R. D. Owen described naturally occurring hematopoietic chimerism for the first time when he observed that freemartin cattle twins shared each other's red cells for long time after birth[2]. Anderson et al further established the presence of chimerism associated tolerance by observing that bovine fraternal twins permanently accepted each other's skin grafts [3]. Billingham, Brent and Medawar created the first experimental tolerance model in neonatal mice where splenic and HSCs from adult mice were injected in neonatal mice. The infused cells when engrafted produced mixed chimerism, and skin grafts from adult mice to neonatal mice survived indefinitely. This seminal work of cell transplantation in a defenseless host became the base for BM transplantation of the future[4]. Slavin demonstrated that such tolerance cannot be induced in adult mice without irradiation [5].

We have achieved prope tolerance in renal transplantation using HSCT [6-9]. We achieved the benefits of activation induced cell death leading to depletion of potentially rejecting T- cell repertoire by using megadose allo-HSCs utilizing Zinkernagel's concept of MHC restriction [10]. We administered donor leucocyte infusions to stimulate donor specific $CD4^+$ clones in the recipient peripheral T-cell repertoire. The next step of non-myeloablative low intensity conditioning with target specific irradiation was taken to create space. Polyclonal anti-T-cell antibodies were used to achieve significant depletion of recipient $CD4^+$ $CD\ 8^+$ cell population to less than 10 % of their baseline levels. Prof. Waldmann has established the critical dose and time factor for this infusion [11]. CsA was administered to protect the chimeric cell population. Cyclophosphamide was used to create space in thymus [12]. Treosulfan along with irradiation was used to delete cobblestone-like stem cell colonies which create allo-resistance to grafting[13]. We adopted the portal route for administration of HSC, based on Gorczynski's demonstration of hepatic chimerism associated donor specific tolerance in mouse model [14]. This technique was developed by us and has been successfully used in all our patients since 1998 [15]. Posselt has created donor-specific unresponsiveness by intrathymic islet cell transplantation [16]. We therefore carried out thymic inoculation of donor HSC to achieve central tolerance by creating thymic chimerism and augmenting apoptotic donor specific clonal deletion. We preferred intra-marrow administration of HSC for better grafting, since the marrow provides an ideal microenvironment for HSC grafting. This method was also devised by us and has been regularly used in all our patients. In majority of our patients we achieved early, adequate stable graft function with almost zero rejection on low dose CsA and steroids, which has been described by Sir Roy Calne as prope tolerance [17].

We observed two cell lineages; $CD3^{dim}$ $CD\ 4^-$ $CD\ 8^-$ and $CD34^+$ $CD\ 33^{weak}$ $CD\ 45^{weak}$, stabilizing from 3.6 ± 1.5 % and 0.09 ± 0.01% respectively at 6 months post-transplantation to 3.8 ± 1.4% and 0.11 ± 0.01% in peripheral blood 1 year post-transplantation. It is not clear what happens to this cell population in other lymphoid tissues; but there is definite increase in the iliac crest of the recipient. We have found stable persistent levels of chimerism for more than 5 years in a subset of our patients. We observed $CD3^{dim}$ $CD\ 4^-$ $CD\ 8^-$ and $CD34^+$ $CD\ 33^{weak}$ $CD\ 45^{weak}$ cell levels of 3.43 ± 1 % and 0.06 ± 0.01% in periphery and 5.9 ± 1.1 % and 0.71 ± 0.01% in BM. This may reflect increased engraftment in the marrow compartment. We have observed that significantly higher level of peripheral NSCC (>3.6 %) correlates significantly better with permanent clinical tolerance than with lower levels. This probably indicates that engraftment has occurred in the marrow. The grafted subset of cells probably originated in the donor BM and was infused in the recipient along with other cells.

We presume that NSC in marrow inoculum facilitate donor specific hypo/ unresponsiveness to alloantigens in the host by getting grafted. NSC from donor BM inoculum will migrate from periphery to the BM and other lymphoid organs. We inoculated donor BM in the recipient BM directly for better grafting in the space already created after irradiation. Presence of such donor cell lineages will lead to a state of mixed hematopoietic chimerism synonymous with unresponsiveness to donor tissue allo-antigens. This is the mechanism of tolerance in our depletion- chimerism human model.

CONCLUSION

Ahmedabad tolerance induction protocol is safe, effective and reproducible in inducing prope tolerance in more than

85 % patients and robust tolerance has been achieved in more than 12 % patients across MHC barriers in LRD renal transplantation. Robust tolerance is achieved with persistent peripheral chimerism of >3.6 % CD3 ^{dim} NSC levels.

FUTURE DIRECTIONS

We plan to develop a method to establish the role of mixed hematopoietic chimerism in thymus for central tolerance induction as an independent denominator. We are also planning to study the depletion phenomenon in the naïve T-cell and memory T-cell compartments as an effect of polyclonal anti-T-cell antibody induced depletion. We plan to evaluate the percentage and role of donor memory T-cells infused, in the reconstituted T-cell repertoire of the recipient.

ACKNOWLEDGEMENTS:

We are indebted to Ms. Priyadarshini Chaudhary, Ms. Nalini Patel, Mr. Yazdi Wadia and Ms. Jyotsana Suthar for updating patient charts, statistical analysis and preparation of the manuscript.

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