Bone marrow augmentation of donor-cell chimerism in kidney, liver, heart, and pancreas islet transplantation

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Summary

We have previously postulated that donor cell chimerism in organ transplantation is needed to attain a tolerant state. Here we show that donor cell chimerism can be augmented in organ recipients if they are infused perioperatively with 3×10^8 per kg of unmodified donor bone marrow cells and are kept on a conventional immunosuppressive regimen of tacrolimus (FK506) and prednisolone.

36 patients took part, of whom the first 18 patients have good transplanted kidney (n=10), liver (n=7), and heart (n=7) function when followed up between 4 and 16 months. All patients are well. We found persistent multilineage leucocyte chimerism in blood of 17 recipients by flow cytometry and PCR techniques to detect donor alleles or Y chromosomes in female recipients of male organs. The use of the 5-antigen HLA matched same sex donor precluded detection of chimerism in one patient.

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Introduction

We have hypothesised that the migration of donor leucocytes from organ transplants and the persistence of these cells in recipient tissues explains allograft acceptance, and[1,2] is the first stage in the development of donor-specific tolerance. We augmented the leucocyte load from the donor by giving a perioperative infusion of unmodified bone marrow to 36 patients. We report the follow-up status of the first 18 patients: 10 kidney recipients, 7 liver recipients, and 1 heart recipient. These patients included 3 diabetics who were also given pancreatic islets intraportally, and a liver recipient with a positive lymphocytotoxic crossmatch.

Patients and methods

Patients

18 followed-up patients treated between December 1992, and December 1993, were aged 20 to 63 years (mean 46, [SD 13·2]) and included 3 diabetics who had been insulin dependent (55–65 units/day) for 18–37 years. The cadaver donors were aged between 8–60 years (mean 31, [SD 14·8]), were ABO identical with their recipients, and had HLA antigen mismatches (of a possible 6) of 3·8 (1·7), 5·7 (0·5), and 6·0 for the kidney, liver, and heart recipients, respectively. No patient had complete HLA compatibility. Patient 6 (a liver recipient) had a positive lymphocytotoxic crossmatch. 9 kidney and 17 liver recipients who did not receive bone marrow because permission for bone removal from the cadaver was denied were used as controls.

Bone marrow preparation*

Thoracolumbar vertebrae from the donors were excised, separated, and transported at room temperature in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, N Y USA). Bone marrow cells from the chipped-off cancellous bone were passively released into the processing medium, filtered, and centrifuged at 300 g for 11 min. The pellet was placed in suspension medium and the cell number and viability by trypan blue exclusion were determined. More suspension medium was added to dilute the cells to 2×10^7/mL, and the final product was refrigerated at 4°C until infusion.

When needed, an aliquot of cell suspension containing 3×10^8 cells/kg, was removed and centrifuged. The pellet was resuspended in 200 mL of the same suspension fluid and infused intravenously over 20 min, 2–12 hours after revascularisation of the whole organ grafts. Samples of the final infusate were retained for dose confirmation, progenitor cell assays, microbial testing, and flow cytometric analysis.

Pancreatic islets

Islet isolation was done with a modified automated method.[3] $5·7 \times 10^5$–$1·5 \times 10^6$ islets ($3·2 \times 10^5$–$8·4 \times 10^5$ islet equivalents) from the organ donor were infused into the portal vein of 1 liver and 2 kidney recipients.

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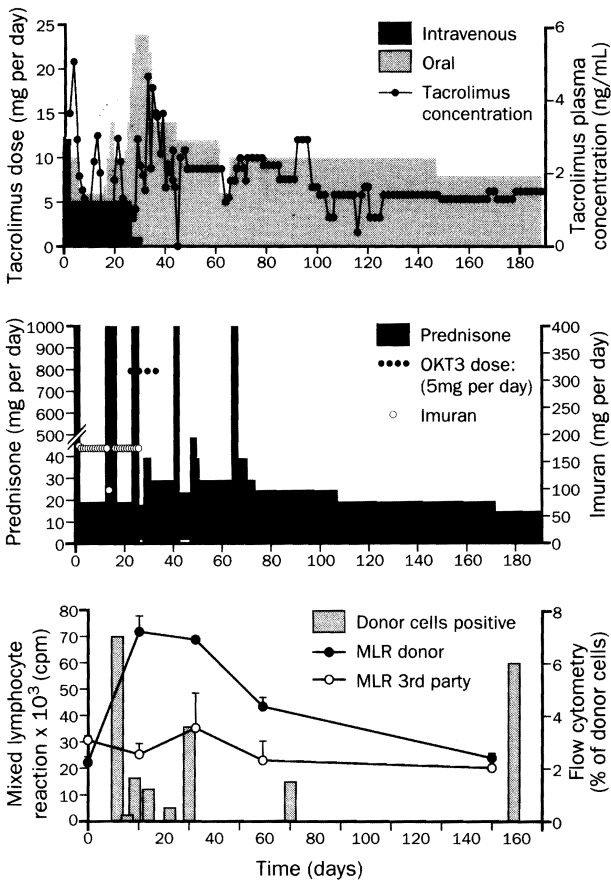*Details of processing and suspension media are kept at *The Lancet.*

Figure 1: **Immunosuppression and MLR responses to irradiated donor and third-party cells, and donor cell chimerism by flow cytometry for case 18 in the first 6 months after heart-bone marrow transplantation.**

Immunosuppression

Routine immunosuppression with Tacrolimus (FK506) and prednisone [4,5] was started after organ revascularisation. Plasma trough concentrations of Tacroliminus were targeted to 1·5–2·0 ng/mL in the first postoperative month, and 0·5–1·2 ng/mL thereafter with adjustments guided by side effects and/or rejection. One gram methylprednisolone (1 g) was given intraoperatively,

followed by a 5-day rapidly tapered prednisone cycle, decreasing from 200 mg to 20 mg by day 6[4,5] except for the heart recipient whose steroid induction was lighter (figure 1). This patient also was given azathioprine for the first 26 days. Drug weaning for the other patients was individually assessed. Azathioprine (n = 3) and OKT3 (n = 1) were reserved for rejection that was not responsive to dose adjustments of Tacrolimus and/or prednisone.

Determination of chimerism

Probes that detected donor leucocyte HLA antigens (chromosome 6) in venous blood were routinely used. Additionally, 4 female recipients of male organs had chimerism detected by Y chromosome in situ hybridisation. Multiple technologies were used to study both the chromosome 6 and the Y chromosome.[2]

Monoclonal antibodies

Primary mouse-anti-human monoclonal antibodies (mAb) directed against HLA class I or II epitopes were labelled by fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse secondary antibodies. Their appropriate specificity and optimal dilution was confirmed by staining donor splenocytes and recipient pretransplant peripheral blood cells. Leucocyte lineages were determined with lineage-specific PE or FITC-conjugated mouse-anti-human mAbs. Isotype-matched irrelevant monoclonal antibodies were used as negative controls.

We sorted and identified donor cells with anti-HLA class I mAb by fluorescent activated cell sorter (FACS) analysis. The reliable detection threshold was 0·5%. Donor and recipient leucocyte lineages were determined in the unsorted cell population and also in sorted donor (positive) and recipient (negative) cells with two-colour immunofluorescence staining and re-analysis by FACS. In cross-control experiments, the DNA was extracted from sorted cells, and the accuracy of their sorting into donor and recipient populations was confirmed by blind examination with polymerase chain reaction (PCR).

In-situ hybridisation

Cytospin preparations of peripheral blood mononuclear cells (PBMC) were hybridised with probes directed against X or Y chromosome at 37°C for at least 30 min. We labelled X-specific probe with digoxigenin and conjugated the Y-specific probe with biotin. The probes were localised by rhodamine-conjugated anti-digoxigenin and FITC-conjugated avidin. Nuclei were counterstained with 4, 6-diamidino-2-phenylindone and viewed using a Nikon Optiphot II microscope fitted with a triple band pass filter. Cytospin and flow cytometry were both used in selected

Patient	Allograft	POD	Graft Function*		Immunosuppression†	H v G (POD)	G v H (POD)	Donor-specific MLR response§ (%) (POD)	Detection of donor cells		
			Bilirubin (mg/dl)	Creatinine (mg/dl)	Tacrolimius/steroid (mg per day)				PODª	FACS (%)	PCR
1	Liver	125	0·5	..	30; 0	None	None	111 (96)	96	4·6	+
2	Liver + islets	198	0·3	..	10; 5	15; 86	None	80 (131)	127	<0·5	+
3	Liver	232	0·8	..	2; 0	7; 22	None	31 (178)	204	1·8	+
4	Liver	251	0·5	..	6; 0	None	54	14 (161)	146	1·9	+
5	Liver	271	0·7	..	8; 0	None	21; 74	107 (236)	236	<0·5	+
6	Liver	294	0·4	..	6; 05	33	None	30 (280)	252	1·6	+
7	Liver	389	1·0	..	2; 0	24	None	28 (368)	265	NF	+
8	Kidney	119	..	1·1	36; 10	None	None	80 (93)	93	0·9	+
9	Kidney	161	..	1·1	20; 12·5	47	None	46 (105)	105	2·3	+
10	Kidney	177	..	1·1	10; 2·5	None	None	28 (134)	..	NF	NF
11	Kidney	243	..	1·6	18;2·5	None	Npone	65 (194)	183	<0·5	+
12	Kidney + Islets	292	..	2·7	9; 15	14; 41; 55;230	None	44 (257)	204	1·3	+
13	Kidney + Islets	294	..	1·4	24; 2·5	16	None	42 (275)	276	3·0	+
14	Kidney	348	..	1·8	6; 0	None	None	18 (316)	316	2·7	NF
15	Kidney	351	..	1·8	18; 10	16; 263	None	13 (323)	232	NF	+
16	Kidney	432	..	1·2	4; 0	16	None	28 (68)	315	<0·5	+
17	Kidney	484	..	1·7	9; 0	None	None	NF	367	NF	+
18	Heart	199	Good cardiac function		8; 15	12–40ᵍ	None	119 (149)	149	6·4	+

POD = Post operative day; HvG = host versus graft; GvH = graft versus host; MLR = mixed lymphocyte reaction; NF = not feasible as no adequate donor spleen cells or cross reactive antibodies.
*C peptide for patients 2, 12, and 13 was 0·02, 0·44, and 0·11 pmol/mL respectively. †Imuran was given to patients 12 and 13 at 75 mg per day. §Percentage of donor specific MLR response compared with the third party on the last sample tested.

Table 1: **Profile of graft function, immunosuppression and in vitro immune status of patients with donor bone marrow and organ transplantation**

patients to determine the congruence of chromosome 6 and Y chromosome studies.

Polymerase chain reaction (PCR)

Previous techniques using PCR to detect spontaneous microchimerism[1,2,6] were feasible in 16 of the 18 cases. It can reliably detect one donor cell within 10 000 recipient cells. In 15 cases, we used probes directed against class II or the Y chromosome. DNA was amplified and resolved by electrophoresis on an agarose gel. After Southern blotting, the membrane was hybridised with a specific radiolabeled probe, exposed to film and developed. In 1 kidney and bone marrow recipient, who was a perfect DR (class II) match with the donor, (case 16, Table 1 and 2), primers specific for the donor HLA class I (A29) allele were used. PCR was not technically feasible in the other 2 cases.

Persistent circulating DNA was much easier to find than in our earlier studies[2,6] (Rudert WA, Kocova M, Rao AS, Trucco M, unpublished) and was quantitated with competitive PCR (cPCR) in 2 female patients who received a male liver (case 4) or kidney (Case 17). The process involved the co-amplification of the sample being tested (the target template) together with an artificial DNA fragment the "competitor"—distinguishing the 2 amplification products by a size difference caused by the addition of a spacer DNA segment in the competitor. By adding known concentrations of competitor DNA to a fixed amount of target template before amplification, we derived the equivalent molar amount of donor genomic DNA in the target sample by matching parallel readings and expressing them as DNA genomic copies.

Immunological testing

The immunological status of the patient pre-transplantation and post-transplantation was determined *in vitro* by the response of patient's PBMC to conconavalin A and phytohaemagluttinen (PHA) mitogens and recall antigen, mixed lymphocyte reaction (MLR), and cell-mediated lymphocytotoxicity (CML). The human MLR cultures were done using gamma-irradiated donor splenocytes and third-party PBMC as stimulators (5×10^4 cells), and with recipient PBMC as responders (5×10^4 cells). The cells were cultured for 6 days in 5% CO_2 in air; for the final 20 hours 37 kbq of [³H]-thymidine was added to each well. We determined ³H thymidine incorporation by liquid scintillation counting. For CML, PHA-activated ⁵¹Cr-labeled donor splenocytes and third-party PBMC were used as targets [T] to assess the effector [E] functions of 5-6 day MLR-cultured recipient's PBMC. We used various E:T ratios, ranging from 10:1 to 40:1.

Results

Clinical course

The bone marrow infusions were uneventful, and convalescence was rapid. The liver-islet recipient developed Epstein-Barr virus-associated lympho-proliferative disease (LPD) limited to the colon on postoperative day 71 which spontaneously stopped when immunosuppression was reduced (Case 2, table 1). All whole organs functioned well after follow-ups of 4 to 16 months. Postoperative C-peptide activity was initially present in all 3 diabetic recipients of pancreatic islets and was high enough in 2 recipients to be insulin-free when Tacrolimus and prednisone (which are both diabetogenic) were stopped. However, the C-peptide diminished below this threshold in 2 of the 3 patients and was at the borderline in the third (0·44 pmol/mL, table 1). All 3 patients remain insulin dependent.

Rejection

Half the patients developed rejection between 7 and 263 days postoperatively (table 1), which was treated with dose adjustments of Tacrolimus and prednisone (n = 6), addition of azathioprine (n = 3), or administration of OKT3. One of
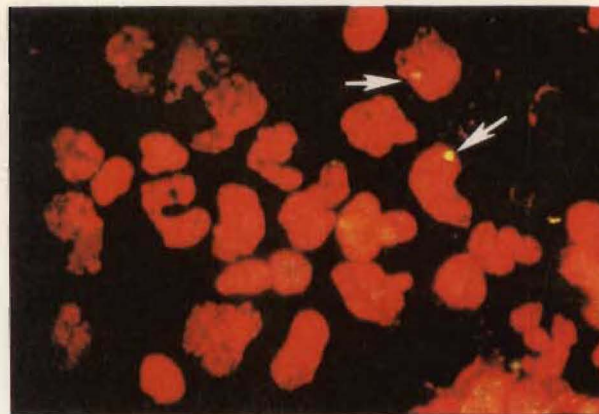

Figure 2: **In situ hybridisation for Y chromosome (arrows) in leucocytes obtained from a female recipient of a male organ (case 11), 120 days after kidney-bone marrow transplantation (× 1000)**

the 3 late rejections (table 1, 86 days) occurred 9 days after discontinuing immunosuppression for the treatment of colonic LPD in a liver-islet recipient. However, the only cause for rejections at 230 and 263 days in kidney recipients was insufficient immunosuppression.

Graft-versus-host disease (GVHD)

Histopathological grade II skin GVHD found on the trunk of one liver recipient was treated by briefly increasing the prednisone dose from 7·5 to 15 mg/day. In another patient a Grade I lesion on the pre-tibial skin resolved spontaneously.

Chimerism

Circulating donor cells were detected in all patients except for a male recipient of a male organ (Case 10) in whom detection was not technically feasible (table 1). Consequently, the chimerism analysis was limited to 17 patients.

In 14 cases in which appropriate monoclonal antibodies were available, 12 (85·7%) had 0·6–5% circulating donor cells in January, 1994, and at the latest examination, 10 (71%) had 0·9–6·4% chimerism (table 1). The other 4 who had no detectable cells with flow cytometry did have the continued presence of donor DNA when PCR methods were used. In the other 3 recipients chimerism was easily demonstrated with Y chromosome detection (figure 2), table 1) or by PCR in all 3 (table 2).

The donor cells quantitated with flow cytometry were multilineage. We verified this observation in a liver-islet recipient who had 1·7% chimerism 82 days post-transplantation, (case 2). Donor cells were sorted, restained for the lineage markers, and reanalysed by FACS. The presence of T, B, natural killer, and progenitor (CD33 or

Patient	Allograft	POD	FACS	Y chromosome detection		
				PCR	cPCR	In situ hybridisation
4	Liver	146*	1·9%	+	1 0%	0·2%
11	Kidney	183†	<0·5%	+	NT	1 0%
15	Kidney	232	NF	+	NT	1·4%
17	Kidney	367	NF	+	0·5%	0·2%

*sample for cPCR and in situ hybridisation was obtained on POD 128; †the sample for in situ hybridization was obtained on POD 120.
NT = not tested; NF = not feasible as cross reactive antibodies

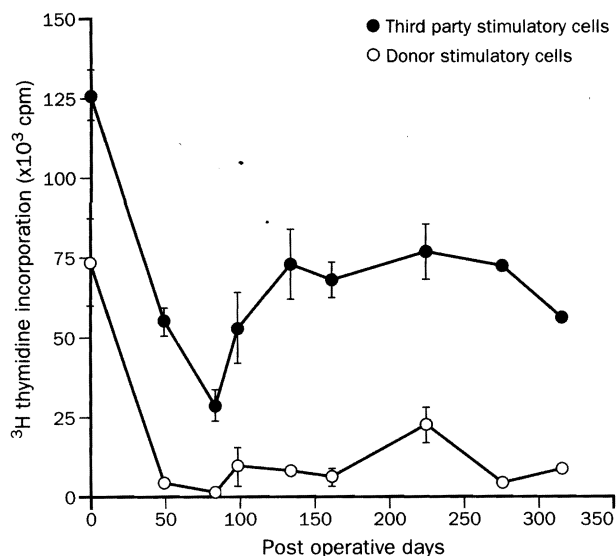Table 2: **Detection of chimerism in 4 female recipients of male organs**

Figure 3: **MLR of a kidney-bone marrow recipient (case 14)**
Mean (SD) of [³H]thymidine incorporation

CD34) cells was confirmed. As a secondary control in patient 2 and patient 11 the cells sorted as donor and recipient were accurately identified as such by blind study with PCR.

Table 2 shows 4 male to female transplant cases. Case 11, had 1·9% chimerism with HLA allele detection (FACS analysis) compared with 1% by the Y chromosome marker method. However, 63 days later less than 0·5% of donor cells were detected by FACS. Patient 4 had quantitative measurement by FACS as well as of the donor genome with cPCR. We demonstrated chimerism in patient 15 with qualitative PCR plus direct demonstration of 1·4% of cells with Y chromosome on day 232 (table 2). Similarly, Y chromosomes were found on day 367 in the cells of patient 17.

PCR detected the highest yield of chimerism (16 of 18 cases). In one recipient a perfect DR match precluded use of a class II oligonucleotide (case 16, table 1). Donor cells were detected up to 315 days with primers specific for the HLA class I allele, A29. Donor DNA in the other 15 patients who were PCR positive was found with a heterotopic HLA class II allele or the SRY region of the Y chromosome until 367 days.

In 2 patients whose Y chromosome was the chimeric marker, donor genome was estimated to be 1·0% of the total DNA at 128 (case 4) and 0·5% at 367 days (case 17, table 2).

	n	Survive >100 days	Graft function (SD)		Chimerism PCR (positive/ total)	Donor-specific hypo-reactivity
			Bilirubin (mg/dL)	Creatinine (mg/dL)		
Kidney						
Without bone marrow	9	8 (89%)	0·5 (0·2)	2·2 (0·9)	3/5	1/5
With bone marrow	10	10 (100%)	0·5 (0·2)	1·6 (0·5)	8/8	5/7
Liver						
Without bone marrow	17	13 (76·5%)*	0·9 (0·4)	1·5 (0·8)	6/11	1/9
With bone marrow	7	7 (100%)	0·7 (0·3)	1·4 (0·4)	7/7	4/7

*of the 17 patients, 4 died within 17–40 days after transplantation due to various causes; after initial follow-up one patient opted to drop out of the study whereas one patient was re-transplanted on POD 21 due to primary graft failure.

Table 3: **Responses of whole organ allograft recipients with and without donor bone marrow augmentation**

Patient 17 was a kidney recipient, and donor DNA gradually disappeared from the blood during the first 2 months but then increased steadily throughout the rest of the first postoperative year.[9] Chimerism was estimated to be 1000 times more than that seen in any of the chronically surviving microchimeric kidney recipients.[8]

Immune monitoring

The lack of donor spleen cells precluded in vitro studies in case 17. In cases 10 and 16 a low donor-specific response preceding and following transplantation meant that meaningful observations were not possible. (Table 1, footnote). Samples from the other patients showed donor-specific hyporeactivity in 60% (table 1), which could be seen as early as 40 to 50 days postoperatively (figure 3).

The remaining 6 patients (cases 1, 2, 5, 8, 11, and 18) maintained vigorous proliferative responses against the donor and panel cells (65–119% of third party) up to the latest sample tested (3 livers, 2 kidneys, and the heart) (table 1). Only 1 patient (case 2) had rejection beyond 40 days. The kidney recipient (case 11) who had pre- and post-transplant donor and third party-specific cytotoxic activity lost the donor-specific killing in CML assays while maintaining third-party killing. There were no other examples of either donor or third-party specific cytotoxic activity before or after transplantation.

Control patients

Table 3 shows survival in the control kidney and liver recipients. The median time to first rejection was 27 days in the 21 control patients who survived through the study compared with 33 days in the marrow-treated patients (not significant, p=0·547 by Mantel-Cox test). Rejection-free interval and cumulative incidence of rejection were the same in the study and the control groups (Kaplan-Meier method). Histopathologically, the rejections were not distinguishable in the control and marrow-augmented groups.

After transplantation, spontaneous chimerism was not detectable by FACS in the control groups, but was found with PCR in 9/16 (56%) of cases where analysis was feasible (6/11 in the livers and 3/5 in the kidneys). Donor-specific hyporeactivity was observed at 4 months in 2/14 (14·3%) of the controls in which testing was feasible versus 9/14 (64·3%) of the bone marrow group at about the same time (table 3).

Discussion

Tolerance induction with live donor leucocytes became the bedrock of modern transplantation after its first description in mice.[9] Pioneer efforts to use tolerance for whole organ transplantation with bone marrow[10] or by using donor specific blood transfusion[11] were hampered by several factors: the assumption that the infused cells have a transient survival unless there is preconditioning; fear that the chimeric cells would cause GVHD; and lack of information about the appropriate timing of leucocyte administration. Consequently, previous leucocyte augmentation trials have failed outright, yielded equivocal results, or been inapplicable to cadaveric transplantation.[11-16] Our discovery that chimerism is a naturally occurring event after whole organ transplantation has enabled us to give MHC-incompatible bone marrow safely without recipient preparation or deviation from standard practices of immunosuppression.

Unaltered bone marrow infusion without recipient preconditioning did not cause perceptible harm to the first 18 patients. The transient GVHD observed in 2 of 7 liver recipients was clinically insignificant and disappeared with little or no change in therapy. The true incidence of these skin manifestations of GVHD in conventionally treated liver or other organ recipients is not available for comparison, because the self-resolving rashes have been incorrectly attributed to transient allergies and not systematically biopsied or treated.[2]

The unusually high 4-month mortality of 11% and 24% for the control kidney and liver recipients, respectively, may have been an artifact of case selection. The severity of rejection in the marrow versus contemporaneous non-marrow cohorts was not qualitatively or quantatively different. The average graft function at 4 months was the same in the culled (by mortality) control and in unculled bone marrow liver groups. The function of the transplanted kidneys in the control patients was poorer than in the experimental series.

The most striking clinical observation was a convalescence pattern of a bidirectional immunological confrontation most clearly defined by rejection (but also by GVHD) that was followed by a successful outcome. These events, which have long been inexplicable, were seen 30 years ago in the first kidney recipients treated with azathioprine and prednisone.[17] The same postoperative events are seen with all transplanted organs and with all immunosuppressive regimens. This characteristic sequence of crisis and recovery is the reason for our original suspicion that persistent chimerism must be present in successful cases.[1] When chimerism was found,[1,2,6,8] the logical next step was to augment it. Our expectation was that the cyclic events of recovery would be much the same as in historical cases but with the ultimate achievement of denser chimerism.

The chimerism with adjuvant bone marrow was 1000-fold greater than that found in our previously reported long-term surviving kidney[8] and liver recipients,[2,6] and greatly exceeded the trace chimerism in the control patients at 4 months. Rejection in 9 (50%) of the bone marrow augmented recipients and minor GVHD in 2 (11%) in the presence of this sustained chimerism underscores our earlier warning that chimerism is not synonymous with tolerance, but is a necessary condition for its attainment.[1,2,18]

Tests of immune reactivity in vitro may help in deciding how long a patient should be kept on continuous suppression. More than half of our test patients have evidence of evolving donor specific nonreactivity by in vitro testing compared with 14% of the non-marrow controls. However all patients still receive immunosuppressive therapy and withdrawal of these agents is not planned until at least 18 months after the operation. While the eventual effect of the augmented chimerism remains speculative, we believe that it will allow our patients to aspire to an eventual drug-free state. Evidence of realistically achieving our objective is reinforced by reports of spontaneously chimeric liver recipients who have not had immunosuppression for many years,[2,19] and smaller numbers of kidney recipients for whom deliberate drug withdrawal is thought to be far more dangerous.[18]

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