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Augmentation of Natural Chimerism With Donor Bone Marrow in Orthotopic Liver Recipients

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WITH the concept that the persistence of donor leukocyte chimerism plays a seminal role in the acceptance of whole organ allografts,[1-4] we initiated a prospective trial in December 1992 to enhance chimerism by adjuvant infusion of unmodified donor bone marrow (BM) in liver, kidney, and thoracic organ recipients.[5] Conventional tacrolimus/prednisone immunosuppression was given without any kind of recipient preconditioning. We report the outcome of the first 34 liver transplantations, along with 29 contemporaneous control cases in which permission could not be obtained for BM removal from the cadaveric liver donors.

MATERIALS AND METHODS

Patients

The 34 transplantations were between March 1993 and December 1994, all from ABO compatible donors. Typing was random and the matching was poor, and in 24% of the cases, the cytotoxic cross match was positive after dithiothreitol treatment (Table 1). A diabetic patient reported elsewhere[5] also received pancreatic islets. The infused BM was not T-cell depleted or modified in any other way, and the recipient was not pre-treated or preconditioned with cytoablative or cytoreductive procedures. Twenty-nine recipients of liver allografts served as controls because permission to remove BM from the cadaveric liver donors could not be obtained. The majority of study (74%) and control (83%) patients were at United Network for Organ Sharing (UNOS) III or IV urgency status at the time of operation. Demographic data are given in Table 1.

Table 1. Demographic Profile of Bone Marrow-Augmented and Nonaugmented Orthotopic Liver Transplant Recipients

Demographic	Bone Marrow Augmented (n = 34)	Bone Marrow Nonaugmented (n = 29)
Age (Yr; x ± SD)		
Recipients	53 ± 9	52 ± 12
Donors	35 ± 16	44 ± 19
Sex (recipient)		
Males	15	14
Females	19	15
Cross-Sex Transplants		
Male → Female	12	10
Female → Male	5	5
Follow-up (d)		
Range	165 to 787	231 to 565
Mean ± SD	398 ± 157	458 ± 90
HLA		
Matches (X ± SD)	1.2 ± 1.2	1.7 ± 1.2
Mismatches (X ± SD)	4.8 ± 1.2	4.3 ± 1.2
Lymphocytotoxic Crossmatch		
Positive	8 (24%)	6 (21%)

BM Processing

BM cells were isolated from thoracolumbar vertebrae with minor modifications from the previously described methods.[5] The separated vertebrae were crunched in addition to the original chipping procedure. The media and process were otherwise the same. In a second alteration, the cells were suspended in lactated Ringer's solution containing 2.5% human serum albumin, 0.5 mg/mL gentamicin, and 10 U/mL heparin (rather than a special "suspension solution") before infusion into the recipients via a central intravenous (IV) line. In a third alteration from the original protocol, the isolated BM cells were filtered (mesh size 180 μ) three times before and after refrigeration and immediately before infusion. This modification was made to avoid the accidental infusion of microscopic bone chips that had been detected in the solution. The dose was 3 to 5 × 10^8 cells/kg body weight, resuspended in approximately 200 mL of the lactated Ringer's solution. A sample of the final infusate was withheld for progenitor cell assays and for microbial testing.

Immunosuppression

Tacrolimus (FK 506, Prograf, Fujisawa Pharmaceutical Co, Osaka, Japan) and prednisone were begun intraoperatively. The daily doses of tacrolimus (beginning at 0.05 IV and 0.20 orally) were targeted to achieve whole blood levels of 10 to 20 ng/mL in the first 1 or 2 weeks and 6 to 12 ng/mL thereafter. However, dose adjustments up or down were individualized from the first day onward, based on side effects and/or allograft function. One liver/BM-recipient with marked pre-transplant encephalopathy did not recover normal neurological function by 17 days (disorientation and delayed responses) and was permanently switched to oral cyclosporine (400 mg/d, Sandoz Pharmaceuticals, East Hanover, NJ). Although dramatic improvement did not occur, he has steadily recovered over a period of 6 months and is out of the hospital.

Prednisone was begun with a one gram bolus intraoperatively, followed with a 5-day cycle (200 → 20 mg). Episodes of acute rejection were treated with a methylprednisolone bolus (1 gm, IV) and a 5-day steroid recycle if necessary. A course of OKT3 (5 mg/d, IV for 7 to 14 days) was given for steroid-resistant rejection, and azathioprine was added if indicated, to control rejection or minimize tacrolimus toxicity. Drug doses were progressively reduced in

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Table 2. Criteria Used to Serially Evaluate In Vitro Mixed Leukocyte Responses of Transplant Recipients

Categories	Proliferative Responses	
	Donor-Specific*	Donor/Third Party
Suppressed†	Markedly reduced	Markedly reduced
Hyporeactive	Decreased (< 20%)	Decreased (60% to 70%)
Intermediate	Decreased (50% to 60%)	Decreased (40% to 50%)
Reactive	No change	No change

*Post- versus pre-transplant responses.
†Nonspecifically diminished response to mitogens as well as alloantigens.

all patients with the usual trial and error establishment of maintenance therapy.

STUDIES OF CHIMERISM

The presence of donor cells in the recipient's peripheral blood mononuclear cells (PBMC) was evaluated weekly in the first month posttransplantation and every other month thereafter.

Flow Cytometry

As reported previously, single and double-color immunofluorescence techniques were used to distinguish donor from recipient cells.[5] After staining, the samples were analyzed using an EPICS Elite Flow Cytometer (Coulter Corp, Hialeah, FL). Unstained and cells stained with appropriate fluorochrome-conjugated isotype-matched irrelevant monoclonal antibodies (MAbs) were used as negative controls. Fifty-thousand (lymphocytes) events were acquired for analysis with open, as well as with gates encompassing the lymphocyte or monocyte populations. In selected cases, recipient PBMCs, stained with various lineage-specific MAbs (CD3, CD22, CD56) were sorted, and the presence of donor DNA was detected subsequently by polymerase chain reaction (PCR) analysis (see below).

PCR

Donor DNA was detected in the recipient's peripheral blood or sorted cells by a procedure described previously.[2,4,5] This technique allowed for detection of as low as one donor/10^5 to 10^6 recipient cells.

IMMUNOLOGICAL RESPONSIVENESS

Pretransplant and serially posttransplant (every other month) monitoring of recipient's immune status was performed by evaluating the proliferative responses of their PBMCs to mitogens (concanavalin A, phytohemagglutinin antigen), recall antigens (tetanus toxoid), mixed leukocyte reactions, and limiting dilution assays. These assays as well as detailed studies of many of these same patients have been described elsewhere.[6] The criteria used to serially evaluate in vitro immune responses is detailed in Table 2.

STATISTICAL ANALYSIS

Comparative analysis of the risk of rejection was performed using the log-rank (Mantel-Cox) test. The cumulative risk of rejection was computed using the Kaplan-Meier (product-limit) method. Risk estimates were determined using the following formula: $1 - S(t)$, where $S(t)$ is the cumulative probability of being rejection-free at time (t). Patients who were lost during the follow-up (ie, died free of rejection) were censored from the final analysis. Continuous variables were compared using the two sample *t*-tests undertaken by using Fisher Exact Test and chi-square test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Clinical Course

No complications of BM infusion were observed in any of the 34 primary liver allograft recipients and their recuperation was rapid. Their follow-up ranged from 165 to 787 days (Table 1). Two liver recipients died on postoperative days 23 and 35 of infections and multiple organ failure. The surviving 32 (94%) patients are well with a mean serum bilirubin level of 0.6 ± 0.2 mg/dL (maximum, 1.2 mg/dL) (Table 3). In addition to tacrolimus (9.1 ± 5.6 mg/d), 3 (9%) are also receiving Imuran (42 ± 14 mg/day). Prednisone has been discontinued in 10 of 32 (31%) of the patients (Table 3).

Of the 29 patients who received liver allografts alone, 4 (14%) have died of various causes within the first year after transplantation. The remaining 25 have optimally functioning allografts (mean bilirubin = 0.7 ± 0.3 mg/dL; maximum, 1.6 mg/dL). These recipients are currently being maintained on variable doses of tacrolimus (7.7 ± 3.4 mg/d), prednisone (3.2 ± 3.7 mg/d), and in 5 cases azathioprine (55 ± 27 mg/d). Prednisone has been discontinued in 13 of 25

Table 3. Current Graft Function and Immunosuppression Profile of Bone Marrow Augmented and Nonaugmented Liver Transplant Recipients

OLT	*n*	Graft Survival*		Bilirubin (mg/dL) (X ± SD)	Tacrolimus (mg/d) (X ± SD)	Steroids (mg/d) (X ± SD)	Patients Off-Steroids	
		n	(%)				*n*	(%)
BM-augmented	34	32/34	(94%)	0.6 ± 0.2	9.1 ± 5.6	5 ± 4.8	10/32	(31%)
Nonaugmented	29	25/29	(86%)	0.7 ± 0.3	7.7 ± 3.4	3.2 ± 3.7	13/25	(52%)

*Actuarial 1-year.
Abbreviations: OLT, orthotopic liver transplantation; BM, bone marrow.

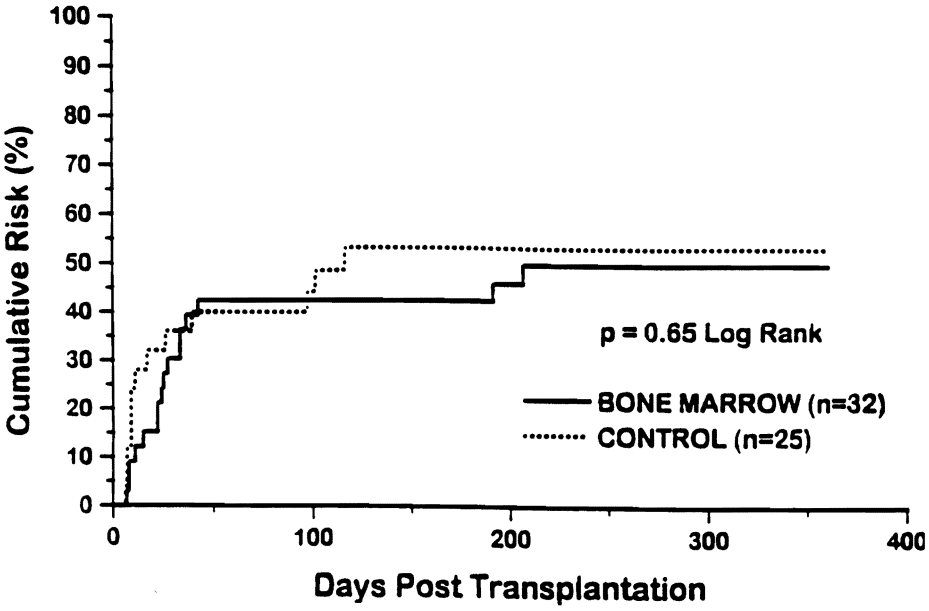

Fig 1. The cumulative risk of rejection in bone marrow-augmented and nonaugmented liver allograft recipients as computed by the Kaplan-Meier (Product-Limit) method. There was no statistically significant difference in the risk between the two groups (P = .65).

(52%) patients, 2 of whom also receive azathioprine (Table 3).

Rejection and Graft-Versus-Host Reaction

Biopsy verified liver rejection followed the same tempo in the BM-augmented and nonaugmented recipients, occurring in 16 of 32 (50%) of study and 13 of 25 (52%) of control patients. The cumulative risk (Fig 1), as well as the histopathological severity of rejection, were similar in both groups, and responded to adjustments of conventional immunosuppression. In 2 of 32 (6%) study and 2 of 25 (8%) control patients, OKT3 was required to treat steroid-resistant rejection.

Histopathologically proven asymptomatic graft-versus-host disease (GVHD), limited to the skin, was observed in 2 of 32 (6%) BM-augmented patients. On postoperative day 54, one patient developed a papulomacular rash (grade II) involving the trunk, which responded to a transient increase in her steroids (7.5 → 15 mg/d). The other patient had two episodes of macular rash (grade < I) on postoperative days 21 and 74, respectively; both resolved spontaneously within 1 to 2 weeks.

Donor Cell Chimerism

Using either PCR (Fig 2) and/or flow cytometry (Fig 3), 30/31 (97%) evaluable BM augmented and 13 of 25 (52%) control patients had evidence of circulating donor cells in their peripheral blood at the last sample tested (Table 4). The unavailability of MAb and/or primers for donor-specific HLA class I or II alleles precluded these studies in one same-sex BM-augmented recipient. The chimerism was of multilineage character (Figs 3 and 4), and the presence of donor T, B, and natural killer cells was confirmed in the recipient PBMCs by PCR studies of a blood sample obtained at 590 days post-transplantation (Fig 4).

In Vitro Immune Monitoring

Because of the lack of recipients' pre-transplant blood and/or donor splenocytes, serial immune monitoring was not possible in 7 of 32 (22%) of the BM-augmented and 2 of 25 (8%) of the control patients. At the last sample tested, a higher (13 of 25; 52%; $P < .05$) proportion of study patients exhibited stable donor-specific hyporeactivity as compared with that of the controls (6 of 23; 26%; Table 5). Vigorous donor-specific immune reactivity was observed in 14 of 23 (60%) of recipients in the control group as compared to 11 of 25 (44%) of those in the study (Table 5). These differences barely achieved statistical significance.

DISCUSSION

The germination of this trial was the realization that the bone marrow-derived multilineage leukocyte component of whole organs (commonly known as passenger leukocytes) begins to migrate ubiquitously within a few minutes after transplantation and that these donor cells survive. They constitute a small second cell population that is capable of defending itself against the immunologic system of the recipient.[1-4,7-9] We have postulated in what has been called the two-way paradigm,[10] that the eventual induction of mutual nonreactivity of the co-existing cell populations, each to the other, is the essential basis of allograft acceptance no matter what the organ.[1-4,7-12].

The ramifications of the two-way paradigm are too vast to consider in this brief report. In this article we have addressed only the narrow issue of augmenting the chimerism emanating from the organ by the simple expedient of adding donor BM. The foremost clinically relevant questions are of safety and efficacy. Concerns about safety have been largely laid to rest, not only by the experience reported here with livers, but also in similar trials in kidney[13] and

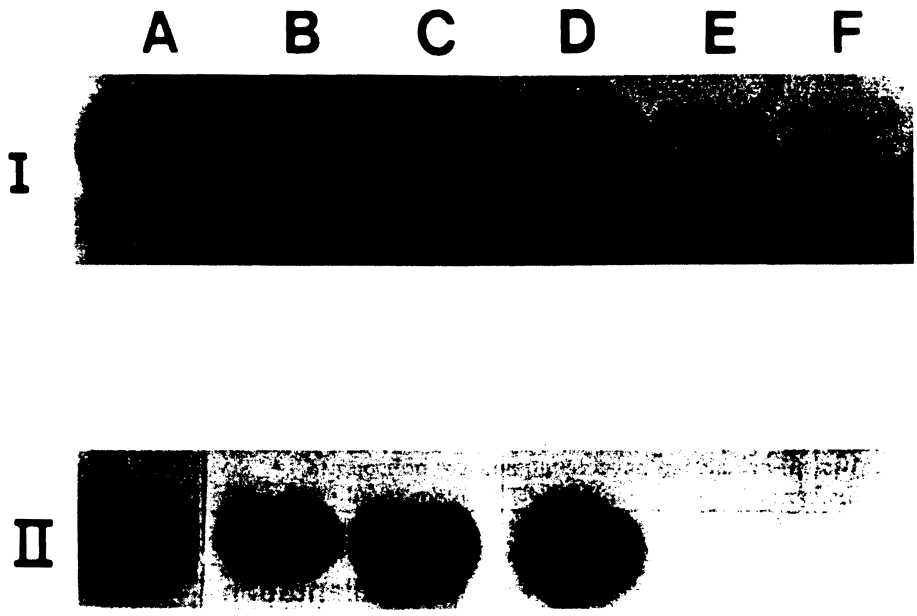

Fig 2. Serial detection of donor DNA in the peripheral blood mononuclear cell of a liver bone marrow recipient for up to 590 days posttransplantation. I: Titration curve of donor-specific HLA class II (DR14); a log dilution from 100 to 0.001 ng in lanes A to E respectively. II: Donor DNA was detectable on POD 252 (lane A), 280 (lane B), 294 (lane C) and 590 (lane D). Lanes IF and IIE are negative controls.

thoracic organ recipients.[14] The clinically insignificant skin rashes in two liver recipients have been the only examples of the once-feared complication of GVHD in the entire Pittsburgh experience.

Although the efficacy question cannot be answered decisively, the extraordinarily high patient and graft survival in this series of high-risk liver transplant recipients is noteworthy. In contrast, our past experience with such cases was faithfully reproduced with the higher mortality of the non-BM control recipients. Not a single patient among the 32 who now have been followed for from 6 months to more than 2 years has the slightest evidence of hepatic graft dysfunction. Similar results in the surviving control recipients bespeak the exceptional efficacy of tacrolimus-based immunosuppression. The chimerism in the BM-augmented patients could usually be detected with flow cytometry as would be predicted from the experimental studies of Murase et al.[9] In Murase's experiments, there was a nearly perfect correlation between the extent of chimerism in rats and freedom from rejection of liver, heart, and other kinds of allografts. The liver produced the greatest chimerism of any organ.

It also was shown by Murase et al[9] that not all donor leukocytes sources are therapeutically equal. When equal numbers of cells were prepared from the thymus and secondary lymphoid organs, only those from BM, with its high proportion of leukocytes of undetermined lineage (presumably including precursor and stem cells), were highly tolerogenic while not causing GVHD. The liver was the most tolerogenic whole organ with little tendency to cause GVHD in the GVHD-prone rat model used in these experiments. In contrast, the lymphoid-rich intestine invariably caused GVHD.[9]

Much more needs to be learned about the reason for these disparities in the effect of leukocytes from different locations. However, circumstantial evidence suggests that a high proportion of dendritic cells, macrophages, and perhaps other cells of myeloid lineage are critical for the desired result of safe graft acceptance. The prominence of chimeric donor dendritic cells in human whole organ recipients as long as 30 years posttransplantation[1-4,12] has directed much attention to this antigen-presenting cell as the key factor in the orchestration of allograft tolerance. Presumably, the outcome in a given circumstance (sensitization versus tolerance) is dependent on the way dendritic cells present alloantigen to the T lymphocyte. Increasing attention has been directed by Steinman, Inaba, and Austyn[15] and by Thomson and Lu et al[16-18] towards the potentially dualistic capabilities of the dendritic cell.

Whatever the mechanisms are proven to be, it should be emphasized that chimerism, although essential for organ allograft acceptance and the induction of donor-specific nonreactivity, is not synonymous with either. In a clinical context, we originally stated that "clinical success—[whether called] tolerance or graft acceptance—means that a characteristic lymphoid and dendritic cell chimerism has been introduced which may be stable either without further treatment, or only when continued immunosuppression is provided; an unstable graft and its migrated cells may either be rejected or cause GVHD."[1]

At a practical level, this statement of principle is hardly an invitation to believe that measuring the level of chimerism will predict when drugs can be stopped or that these levels should be used to guide drug dosing. Although complete drug discontinuance has been frequently achieved long after the transplantation of non–BM-augmented liver recipients,[4] Ramos et al[19] have provided a road map with warning signs for those who would aspire to premature

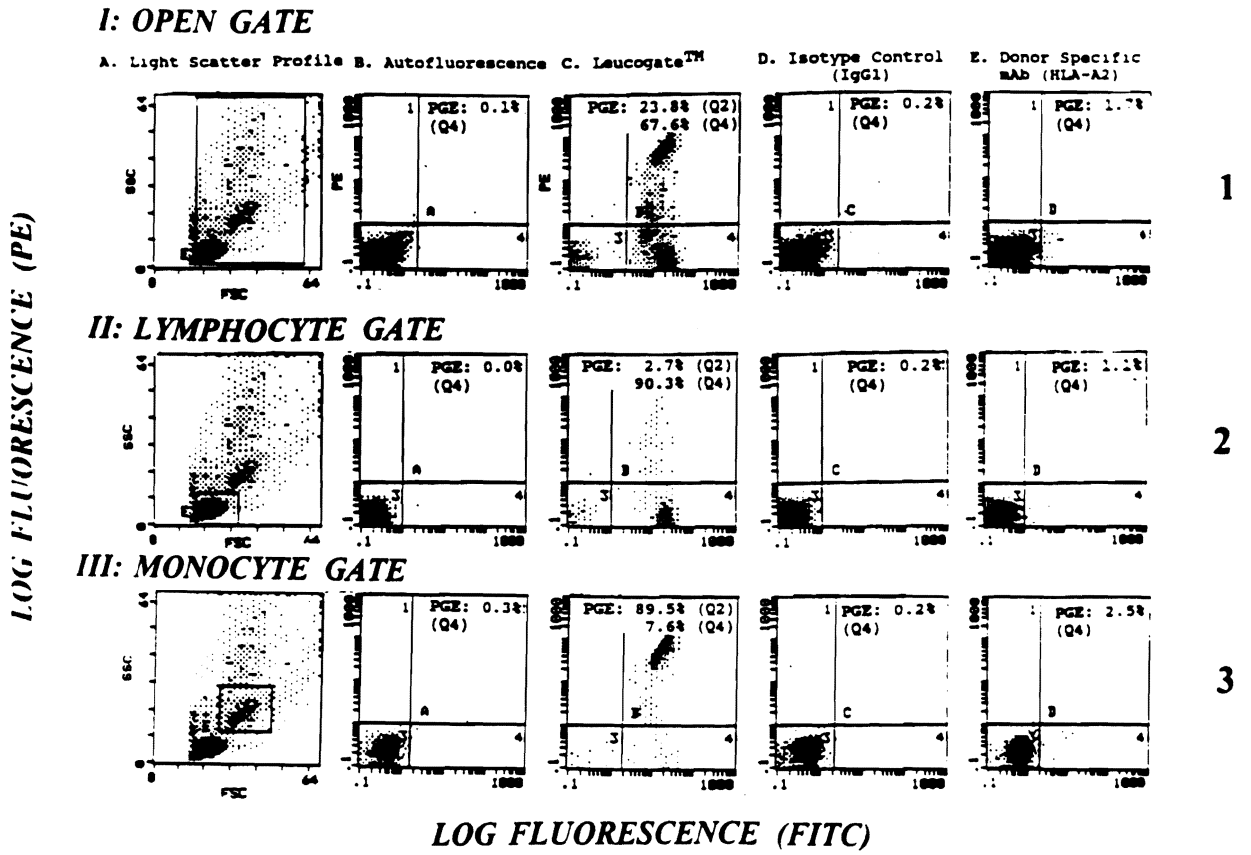

Fig 3. Detection of HLA-A2$^+$ donor cells by flow cytometry in a liver and bone marrow recipient using wide analysis gate (IA). One point seven percent of circulating donor cells (IE) were identified in the recipients' peripheral blood mononuclear cells obtained on postoperative day 380. Furthermore, analysis of cells within the lymphocyte (IIA) or monocyte (IIIA) gates also revealed the presence of 1.1% (IIE) and 2.5% (IIIE) of donor cells, respectively, confirming the multilineal nature of donor-cell chimerism. All analyses were performed on an EPICS Elite Flow Cytometer (Coulter Corp, Hialeah, FL).

weaning.[20] In our drug weaning trials, no liver recipient has been considered for the final step of elective drug stoppage unless they were free of immunologic complications for at least 5 to 10 years.

The observations reported herein hold promise of expanding our grasp of human immunology, while at the same time improving patient care. For example, while Monaco and Wood have long contended that organ allograft acceptance could be facilitated by giving donor BM,[21] it was generally thought that these cells were short-lived. Human trials of BM-augmentation for renal transplantation by Monaco[22] and Barber,[23] as well as for liver transplantation by Rolles[24] have provided equivocal or disappointing results; our results so far should be viewed as equivocal. Until recently, fate of the injected cells was unknown but assumed to be of limited duration. For this reason, it was logical to judge therapeutic efficacy on the basis of early posttransplantation events such as the incidence and severity of acute rejection or short-term patient and graft survival. The experimental studies of Murase et al[9] and Shin et al[25] have made it clear the tolerogenic effect of histoincompatible donor leukocytes, originating either from the graft, or given separately as with BM-augmentation, has the long-term objective of preventing chronic rejection. It seems obvious

Table 4. In Vitro MLR Responses in Bone Marrow–Augmented and Control Patients*

OLT	Evaluable		MLR Responses (*n*, %)†		
	n	(%)	Hypo/Intermediate (%)	Reactive (%)	Suppressed (%)
BM augmented	25/32	(78%)	13/25 (52%)‡	11/25 (44%)	01/25 (04%)
Controls	23/25	(92%)	06/23 (26%)	14/23 (61%)	03/23 (13%)

*At the last sample tested.
†See Table 2 for details regarding the criteria used to categorize responses.
‡Bone marrow-augmented versus controls; $P < .05$.
Abbreviations: MLR, mixed lymphocyte reaction; OLT, orthotopic liver transplantation; BM, bone marrow.

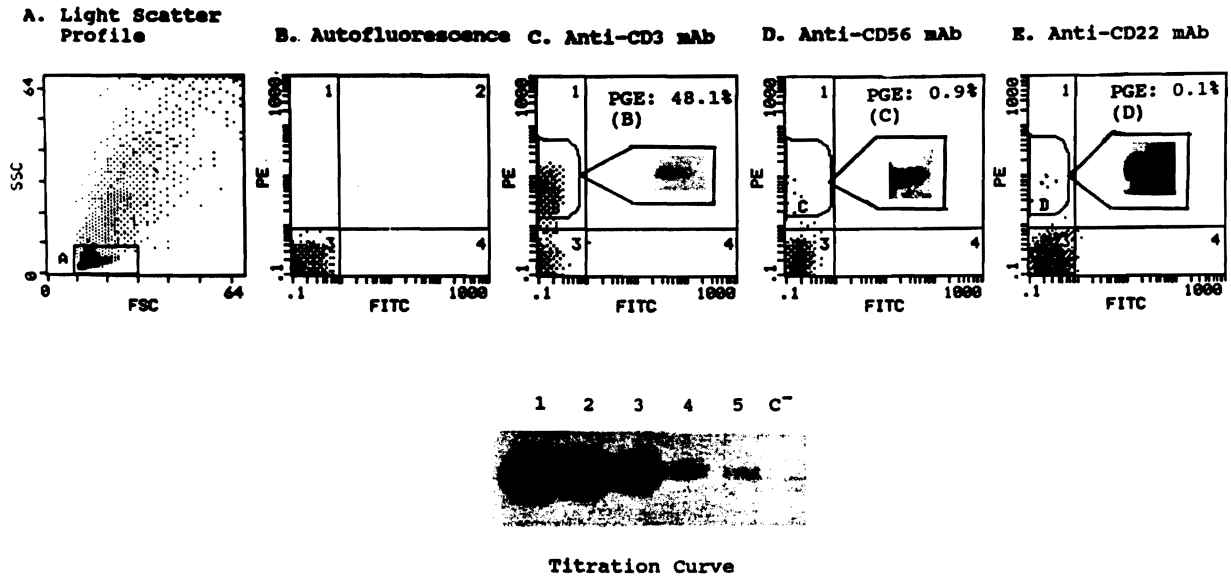

Fig 4. Analysis for the lineage of donor-cells in the peripheral blood mononuclear cell of a bone marrow–augmented liver recipient 590 days posttransplantation. Using lineage-specific monoclonal antibodies, T(C), NK(D) and B(E) cells were sorted, and the presence of donor DNA (inset) within the sorted populations were confirmed by polymerase chain reaction. (B) Autofluorescence of unstained cells. (C) Negative control. Titration curve for DR14 (donor-specific HLA) at log dilution from 100 ng (lane 1) to 0.001 ng (lane 5). PGE: percentage gated events.

that the donor-specific blood transfusion effect, first reported by Salvatierra et al,[26] is a variation of the same principle of chimerism as was postulated by van Twuyver et al.[27]

In conclusion, in an attempt to augment the spontaneous donor cell chimerism which we have postulated to be the explanation of allograft acceptance, we infused 3 to 5 $\times$ 10^8 unmodified donor BM cells/kg body weight into 34 adult primary liver allograft recipients (53 $\pm$ 9 years old) for whom mean follow-up is 398 days (range, 165 to 787 days). Mean donor age was 35 $\pm$ 16 years. Routine immunosuppression was with tacrolimus and prednisone. There were no complications of BM infusion. Two patients (one with a positive cytotoxic cross match, 1:1,024) died of infections and multiple organ failure after 23, and 36 days, respectively. All 32 (94%) surviving patients are well with a mean serum bilirubin level of 0.6 $\pm$ 0.2 mg/dL (maximum, 1.2 mg/dL). Sixteen (50%) of the 32 long-surviving patients had mild to moderate rejection which was responsive to conventional secondary adjustments of immunosuppression (two requiring OKT3). Two patients with asymptomatic skin rash were shown by biopsy to have histopathologic findings of grade 1–2 GVHD disease which involuted spontaneously in one, and after a transient increase in prednisone (7.5 $\rightarrow$ 15 mg/d) in the other. Prednisone has been discontinued in 10 of 32 (31%) of the patients. Donor blood cell chimerism was detected in 30 of 31 (97%) evaluable patients with flow cytometry and/or PCR. In 11 (57%) of 25 evaluable recipients, stable donor-specific hyporeactivity was shown with in vitro testing. Twenty-nine recipients for whom BM was not available because of failure to obtain donor family consent made up a contemporaneous control group. Four (14%) died of various causes during the 231- to 565-day follow-up period. Low-level chimerism was shown in 13 (52%) at last sampling. Six (26%) of 23 evaluable patients exhibited donor-specific hyporeactivity by the time of the last blood sampling. This experience indicates that adjuvant infusion of BM for liver allograft recipients is safe and augmented the level of chimerism. There was a trend towards more frequent development of stable donor-specific nonreactivity in the BM-augmented patients than in the controls.

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