

Effects of Donor Bone Marrow Infusion in Clinical Lung Transplantation

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Background. We have demonstrated that donor cell chimerism is associated with a lower incidence of obliterative bronchiolitis (OB) in lung recipients, and that donor chimerism is augmented by the infusion of donor bone marrow (BM). We herein report the intermediate results of a trial combining the infusion of donor BM and lung transplantation.

Methods. Clinical and in vitro data of 26 lung recipients receiving concurrent infusion of donor bone marrow (3.0 to 6.0×10^6 cells/kg) were compared with those of 13 patients receiving lung transplant alone.

Results. Patient survival and freedom from acute rejection were similar between groups. Of the patients whose

graft survived greater than 4 months, 5% (1 of 22) of BM and 33% (4 of 12) of control patients, developed histologic evidence of OB ($p = 0.04$). A higher proportion (but not statistically significant) of BM recipients (7 of 10, 70%) exhibited donor-specific hyporeactivity by mixed lymphocyte reaction assays as compared with the controls (2 of 7, 28%).

Conclusions. Infusion of donor BM at the time of lung transplantation is safe, and is associated with recipients' immune modulation and a lower rate of obliterative bronchiolitis.

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We and others have reported that a low level of bone-marrow derived cells was detectable in the peripheral blood and tissues of long-term survivors of liver [1], kidney [2], heart [3], lung, and heart-lung [4] allografts. This phenomenon of donor cell chimerism, which occurs by seeding of the host's tissues with cells from the graft [5], was associated with a lower incidence of chronic rejection in lung recipients [4]. To augment donor cell chimerism, in order to modulate the response of the recipient to the allograft, we initiated a prospective trial combining the infusion of donor bone marrow and lung transplantation. Reported herein is the intermediate outcome of this clinical trial.

Patients and Methods

This study was approved by the Institutional Review Board of the University of Pittsburgh on July 14, 1993, and informed consent was obtained from all patients. Between September 1993 and July 1998, 26 adult patients received combined infusion of donor bone marrow and lung transplant. In addition, unavailability of consent to retrieve donor bone marrow has resulted in the accrual of 13 contemporaneous control patients, who received lung

transplant alone. These patients, all undergoing primary lung transplantation, were not conditioned by myeloablative or myeloreductive regimen before transplantation.

Bone Marrow Preparation and Infusion

Donor bone marrow cells were isolated from thoracolumbar vertebrae as described [6]. Unmodified bone marrow cells, at a dose of 3.0 to 6.0×10^6 cells/kg of recipient's body weight, were resuspended in 200 mL of the suspension medium, and infused into the patient within 2 hours after preparation, and between 6 to 10 hours after revascularization of the heart.

Immunosuppression

Immunosuppression consisted of tacrolimus (FK506, Prograf; Fujisawa USA, Deerfield, IL) and steroids, as previously described. During the first postoperative month, the dose of tacrolimus was targeted to maintain whole blood trough levels of 15 to 20 ng/mL. Depending on the side effects and history of rejection, tacrolimus dose was gradually reduced to achieve levels of 10 to 15 ng/mL. Methylprednisolone (500 mg; Upjohn Pharmaceuticals, Kalamazoo, MI) was given intraoperatively before revascularization of the lung graft. Subsequently, a short course of steroid cycle was initiated on postoperative day (POD) 0, starting with 200 mg of methylprednisolone per day administered intravenously in 4 divided doses. The dose of methylprednisolone was tapered by a daily decrement of 40 mg/day and converted to oral pred-

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nisone, (20 mg/day) on POD 5. Systematic reduction of prednisone dose (by 2.5 to 5 mg decrements) was initiated in all patients 3 months after transplantation, if there was no significant rejection by transbronchial biopsy. Azathioprine (2 mg/kg/day; Imuran; Burroughs Wellcome, Research Triangle Park, NC) of mycophenolate mofetil (2 g/day; Cell-Cept; Roche Laboratories, Basel, Switzerland) was added if there was recurrent rejection, or when renal dysfunction (serum creatinine > 2.0 mg/dL) necessitated a reduction of tacrolimus dose.

Monitoring and Treatment of Rejection

Surveillance bronchoscopy and transbronchial biopsy was performed in all patients between POD 14 and 21, unless clinical criteria warranted earlier intervention. Subsequently, surveillance biopsies were obtained every 3 months in the first year, and every fourth month in the second year. Thereafter, the biopsy schedule was dictated by clinical symptoms, and by results of pulmonary function tests. Follow-up biopsies are generally performed 3 to 4 weeks following treatment of rejection, or after cytomegalovirus pneumonia. Acute rejection was defined by histologic criteria [7], with grade II or higher considered significant, and required treatment. Previously described criteria were used for the histological diagnosis and clinical staging of obliterative bronchiolitis (OB) [8, 9]. The histological diagnosis for OB was made in a blinded fashion. Acute rejection was treated with pulses of methylprednisolone (1 g/day for 3 consecutive days). Cytolytic therapy was used when the rejection was refractory to 2 to 3 courses of pulse steroids.

Detection of Chimerism

After transplantation, recipients were typed for donor chimerism in peripheral blood leukocytes by flow cytometry, as previously reported [6]. Blood samples (20 mL) from the patients were obtained on day 0 (time of transplantation), day 15, day 30, day 60, and then every other month during the first 2 years after transplantation, for the detection of donor cells. After staining with the appropriate antibody against donor MHC class I antigens, single-color fluorescence-activated cell sorting (FACS) analysis was performed to identify donor cells, using an EPICS Elite Flow Cytometer (Coulter Corp, Hialeah, FL). Fifty thousand events were collected per sample for analyses. Values of circulating donor cells of less than 0.5% were considered not quantifiable.

Immune Monitoring

Pretransplant and serial posttransplant (every other month) monitoring of recipients' immune status was carried out by evaluating the proliferative responses of their peripheral blood leukocytes to mitogens (concanavalin A, phytohemagglutinin), and mixed leukocyte reactions (MLR), as previously described [10]. Recipients' donor-specific MLR responses (D) at various times posttransplantation were compared to the recipients' pretransplant donor-specific responses, and to responses to cells from third party controls (TP). Donor-specific reactivity was classified according to the previously described

criteria [10]. Briefly, donor-specific hyporeactivity (category I) was defined as at least a 70% decrease in posttransplant versus pretransplant donor-specific MLR responses, while maintaining reactivity to both third party stimulators (D/TP ratio < 40%) and to mitogens (> 50% of pretransplant responses). Donor-specific intermediate reactivity (category II) was designated when there was a 40% to 70% inhibition of antidonor activity with retention of third party responsiveness, whereas reactive (category III) meant that there was minimal or no decline in donor-specific nonreactivity. Suppression (category IV) connoted a nonspecific diminished proliferative response to mitogens as well as to alloantigens.

Statistical Analysis

Continuous variables were expressed as mean \pm standard deviation (SD), and compared using *t*-test or Mann-Whitney test when appropriate. Differences in proportions were compared using the χ^2 or Fisher exact test. Survival and freedom from acute rejection were estimated by Kaplan-Meier method, and compared using the log-rank test. A *p* value less than 0.05 was considered statistically significant. A software package (CSS Statistica, Release 4.5; Statsoft, Tulsa, OK) was used for statistical analyses.

Results

Clinical Course and Patient Survival

The infusion of donor bone marrow was well tolerated. None of the 26 BM recipients developed graft-versus-host disease or had complications related to the infusion of donor bone marrow. The 1 and 3-year actuarial patient survival rates were 77%, 77% for the controls, and 81% and 77% for the BM group, respectively (*p* = 0.9). Seven patients in the BM group died from bacterial infection (*n* = 2), OB (*n* = 1), fungal infection (*n* = 1), renal failure (*n* = 1), cerebral bleeding (*n* = 1), and cardiac arrest (*n* = 1). In the control group there were 5 deaths, and the causes include bacterial infection (*n* = 1), OB (*n* = 1), respiratory failure (*n* = 1), pancreatitis (*n* = 1), and multisystem organ failure (*n* = 1).

Acute Rejection and Obliterative Bronchiolitis

The linearized rejection rates (episode per patient) during the first 6 months after transplantation were 2.6 ± 0.3 and 2.0 ± 0.2 in the BM and control groups, respectively (*p* = 0.5). Only patients who survived for at least 6 months after transplantation were included in the calculation of the linearized rejection rate. Freedom from acute rejection at 100 days after transplantation (by Kaplan-Meier method) was 25.0% in the bone marrow group and 8.0% in the control (*p* = 0.9).

Of the patients whose graft survival time was greater than 4 months, 5% (1 of 22) bone marrow patients and 33% (4 of 12) control patients developed histological evidence of OB on transbronchial biopsy (*p* = 0.04). Two patients in the bone marrow group (2 of 22) and 2 in the control group (2 of 12) have clinical bronchiolitis obliter-

Table 1. Prevalence of Pathological Obliterative Bronchiolitis and Clinical Bronchiolitis Obliterans Syndrome

Patient Group	Bone Marrow	Control	p Value ^a
Surviving > 4 months	22	12	
With OB	1 (5%)	4 (33%)	0.04
With BOS	2 (9%)	2 (17%)	0.60
With either OB or BOS	2 (9%)	5 (42%)	0.07

^a Two-tailed, Fisher exact test.

BOS = bronchiolitis obliterans syndrome; OB = obliterative bronchiolitis.

ans syndrome (BOS), and one in each group had both OB and BOS. In other words, the proportion of patients with either OB or BOS in the bone marrow and control groups was 9% (2 of 22) and 42% (5 of 12), respectively ($p = 0.07$; Table 1).

Immunosuppression Profiles

At last follow-up, or at the time of death, the dose and level of tacrolimus, the dose of prednisone, and the number of patients on prednisone were similar between the two groups (Table 2). However, the number of patients requiring a third drug (either azathioprine or mycophenolate mofetil), because of persistent rejection or renal dysfunction (defined as having a serum creatinine > 2 mg/dL), is higher in the control group (77% [10 of 13] versus 17% [4 of 23], $p = 0.0009$).

Donor Chimerism

Detection of donor cells was feasible (when appropriate antibody to donor HLA antigens were available) in 13 BM patients and 8 controls. The numbers are too small for meaningful statistical analyses, however there is trend toward higher level of donor chimerism in the peripheral blood of BM patients, as compared with the controls (Fig 1, 2). In both groups the level of donor chimerism is higher in the early postoperative period, and decreases with time. However, the chimerism persists much longer in the BM group than in the control. None of the control group had detectable level of donor chimerism at 1 year, whereas 63% (5 of 8) of BM recipients still had detectable

Table 2. Immunosuppression Profiles^a of Controls and Lung Recipients Receiving Bone Marrow Infusion

Immunosuppression	Bone Marrow	Control
FK dose (mg)	10.8 ± 7.1	8.2 ± 3.6
FK level (ng/mL)	13.3 ± 5.3	12.0 ± 3.9
Prednisone dose (mg)	8.3 ± 4.3	6.8 ± 2.3
Patients on prednisone	18/23 (78%)	12/13 (92%)
Patients on third drug	4/23 (17%)	10/13 (77%) ^b

^a Only in patients surviving more than 1 month after transplantation.

^b $p = 0.0009$, two-tailed, Fisher exact test.

FK = tacrolimus.

Table 3. Demographics of Lung Recipients

Variables	Bone Marrow	Control
n	26	13
Recipient age (y)	48.1 ± 11.0	50.23 ± 8.0
Donor age (y)	25.3 ± 10.9	30.9 ± 10.1
Sex (M:F)	18:8	3:10 ^a
Type of transplant		
Single lung	19.0	9.0
Double lung	7.0	3.0
Heart lung	2.0	1.0
Donor ischemic time (min)	235.1 ± 76.2	279.6 ± 51.0
MHC mismatch	4.8 ± 1.0	4.9 ± 1.0
Follow-up duration (days)	729 ± 475	841 ± 445

^a $p < 0.05$, Fisher's exact test.

MHC = major histocompatibility complex.

level of chimerism (by flow cytometry) more than 1 year after transplantation.

In Vitro Immune Testing

In vitro immune testing was possible only when donor splenocytes were available. Recipients' donor-specific, mixed leukocyte responses to donor antigens at various times posttransplantation were compared with the recipient's pretransplant responses. Using previously described criteria [10] we found that, a higher proportion (but not statistically significant, $p = 0.15$) of BM recipients (7 of 10; 70%) exhibit donor-specific hyporeactivity as compared with the control group (2 of 7; 28%). Figure 3 depicts the profile of a mixed leukocyte response of a BM-lung recipient who demonstrated donor-specific hyporesponsiveness.

Comment

The use of bone marrow-derived cells (splenocytes) to achieve donor-specific transplantation tolerance in neonatal mice was first reported by Billingham, Brent, and Medawar [11]. Subsequently, chimerism and donor-specific transplantation tolerance was achieved in adult animals by preconditioning the host with different regimens which include, among others, total body irradiation [12], total lymphoid irradiation [13], and the use antilymphocyte globulin [14]. The clinical use of donor bone marrow to prolong the survival of organ allograft was first attempted in kidney transplant recipients. Monaco and colleagues [15] were the first to report the use of antilymphocyte globulin induction and delayed (25 days after organ transplantation) donor bone marrow infusion in a kidney transplant recipient. Subsequently, Barber and associates adopted the approach of Monaco, and preconditioned a series of renal transplant recipients with antilymphocyte globulin, cyclosporine, prednisone, and azathioprine before infusion of donor bone marrow, and demonstrated better graft survival and less acute rejection in the bone marrow group [16]. Low levels of donor chimerism were detected (by PCR) in approxi-

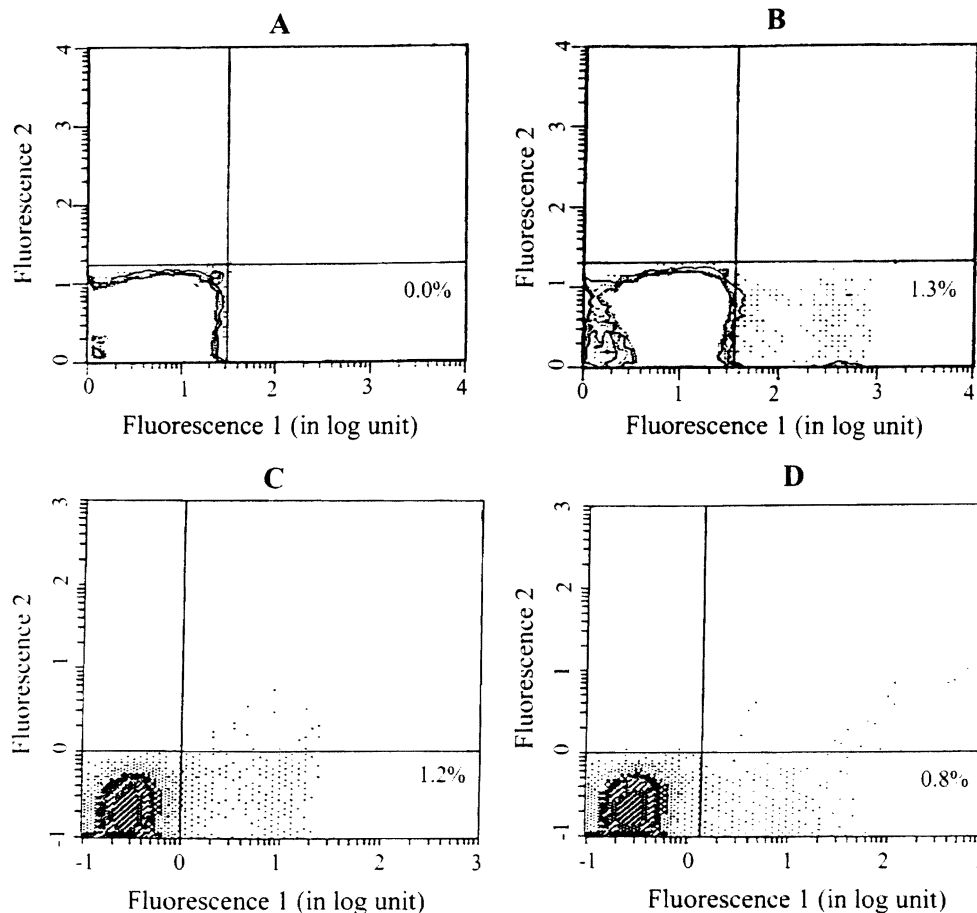


Fig 1. Donor chimerism in the peripheral blood leukocytes of a lung recipients who received bone marrow infusion. Recipient cells were stained with a primary mouse antibody against human MHC class I, then counterstained with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody, and analyzed using an EPICS Elite Flow Cytometer (Coulter Corp, Hialeah, FL). (A) Before transplantation; (B) 1 month, (C) 6 months, and (D) 9 months after transplantation. The number in the right lower quadrant indicates the percentage of donor cells. The level of chimerism was higher after transplantation, but dwindled with time.

mately half of the bone marrow group [17]. In another study, Kahn and colleagues used high dose total lymphoid irradiation (5,400 to 6,000 cGy) to precondition a

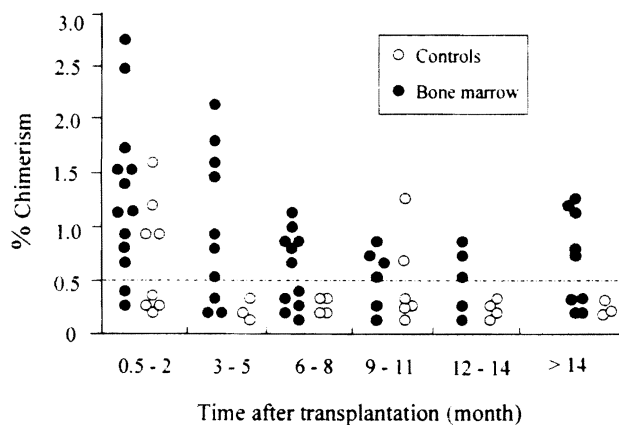


Fig 2. Donor cell chimerism detected by flow cytometry in lung recipients. Each circle represents 1 patient. Level below 0.5% is considered not quantifiable.

series of heart recipients before heart transplantation and intraperitoneal injection of donor bone marrow [18], and reported high perioperative death from infection. Of note is the fact that in these trials antilymphocytes globulin, and radiation had been used to precondition the recipients before bone marrow infusion.

The scientific rationale for the current study, which did not involve preconditioning of the recipient before bone marrow infusion, was based on the discovery by Starzl and associates, that donor cells of bone marrow origin persisted at low level in peripheral blood, lymphoid organs, and skin of long-surviving liver and kidney recipients [1, 2]. Based on these observations, we posited that donor cell chimerism was perhaps essential for the long-term allograft acceptance. Therefore, we hypothesized that augmenting this spontaneously occurring event with perioperative donor bone marrow infusion may further enhance the acceptance of the graft, especially of those organs which are not endowed with a large quantity of passenger leukocytes. To test this hypothesis, in December 1992, we initiated a trial combining donor bone marrow infusion with solid organ transplantation,

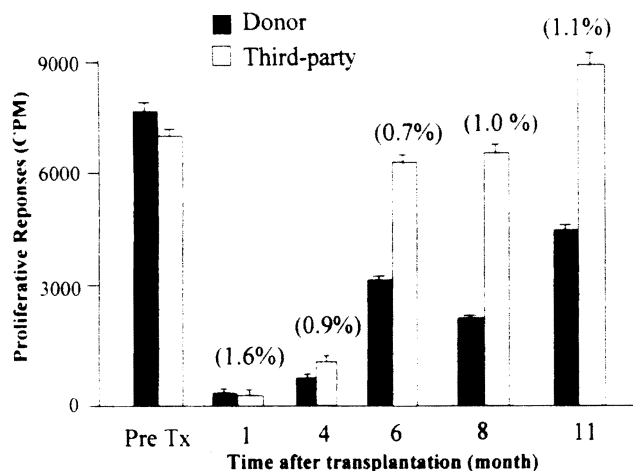


Fig 3. Mixed lymphocyte response demonstrating donor-specific hyposensitization lung recipient who received donor bone marrow infusion. Freshly isolated recipient peripheral blood leukocytes were used as responders, while γ -irradiated donor splenocytes and third-party cells were used as stimulators. The response to donor stimulators was lowered than that to third-party stimulators. Numbers in parentheses indicate the level of chimerism (by flow cytometry).

without preconditioning of the host [6]. Our aim was to augment this *de novo* phenomenon (chimerism) with the hope to reduce the incidence of rejection.

The preliminary results in lung recipients reported herein, indicate that the infusion of unmodified donor bone marrow concurrently with lung transplantation is safe, and is associated with a trend towards higher level of donor cell chimerism, and less donor alloreactivity (by MLR assay). Patients in the bone marrow group had less requirement for immunosuppression (17% of the BM patients required a third drug beside tacrolimus versus 77% of the controls), lower incidence of obliterative bronchiolitis, at least by histologic criteria, than the control. One of the limitations of the current study is its small sample size and its short follow-up.

Following our initial report [6], the transplant group at the University of Miami initiated a series of studies using single and multiple infusions of donor bone marrow in kidney and liver recipients. Our results in lung recipients reported herein are in agreement with the data on kidney recipients from this group. Garcia-Morales and colleagues [19] studied 40 kidney recipients who received unmodified donor bone marrow infusion, and 100 controls who received kidney alone. Their immunosuppression protocol included OKT3 induction therapy, tacrolimus, and steroid maintenance therapy, and in some patients, mycophenolate mofetil. The authors used a newly developed PCR-flow assay (a combination of PCR and flow cytometric techniques that detect donor versus recipient histocompatibility genes as well as cell surface CD epitope markers) to measure donor cell chimerism in the recipient's peripheral blood lymphocytes (PBL) and bone marrow. The bone marrow recipients have higher level of donor chimerism than the controls. Notably, the level of donor chimerism (especially CD3+ and CD34+

cells) was 10-fold higher in the bone marrow compartment than in the peripheral blood leukocytes. Immunologically, the bone marrow patients displayed more depressed humoral and cellular immune responses than the controls. Recipients who were HLA-DR identical with their donors had a high level of chimerism, and no acute rejection. In their most recent update [20], these investigators analyzed the results of 63 cadaveric kidney recipients who received either one ($n = 21$), or two ($n = 42$) infusions of donor bone marrow and 220 cadaveric kidney recipients who did not receive bone marrow (controls). Although there was no difference in the rates of acute rejection, the incidence of chronic rejection in the bone marrow group was lower than the control ($p < 0.02$). The dose of bone marrow cells and the timing of their infusion appear to influence the immune modulation of the hosts. Ricordi and colleagues administered donor bone marrow to liver transplant recipients at varying schedules after transplantation [21]. Patients receiving multiple infusion of bone marrow cells had significantly longer graft survival than those receiving a single infusion.

Collectively, the results of the present study, along with other clinical trials in which donor bone marrow cells were infused into recipients of solid organs, suggest that donor bone marrow cells may have a modulatory effect on the recipient's immune systems, resulting in a salutary impact on chronic allograft rejection. While the long-term effect of the donor bone marrow infusion in lung recipients remains speculative, because of the small sample size and relatively short follow-up duration, it is conceivable that presence of donor chimerism will enhance the acceptance of the graft and reduce the incidence of chronic rejection. Future study with larger sample size and longer follow-up will clarify this issue.

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DISCUSSION

DR JOHN H. CALHOON (San Antonio, TX): My thanks to Dr Pham and his colleagues from Pittsburgh on a nicely presented series, which should cause us all to reevaluate our lung transplant practice. One question to Dr Pham, about a manuscript and presentation that was very complete and well done, why do you think there was a difference in the biopsy incidence of obliterative bronchiolitis and the incidence of true bronchiolitis obliterans syndrome?

The other question would center around, where do we go from here? What do you think the optimum time of donor marrow transfusion would be? How would you best augment the engraftment? Would you use cytolytics, consider total lymphoid irradiation, the amount of infusion and/or, as I mentioned, its timing?

I would like to thank Dr Pham and his colleagues for this presentation and its contribution to our Society and profession.

DR FREDERICK L. GROVER (Denver, CO): I would like to particularly thank Dr Pham for his many years of contributions to the literature, and to our knowledge in the area of transplantation tolerance and chimerism. I think the most distressing part of taking care of lung transplant patients is the 30% to 50% incidence of the development of chronic obliterative bronchiolitis, which often leads to death, and any light that can be shed in this area is obviously very important.

I was particularly interested in those patients who received bone marrow infusion, but failed and later developed evidence of chronic rejection or obliterative bronchiolitis. Did those specific patients show decreased reactivity or did they not keep sustained chimerism?

And finally, how practical is the use of this? What percentage of patients will ultimately consent for infusion of the bone marrow?

Again, thank you very much.

DR PHAM: Thank you very much, Dr Calhoon for your kind comments. As I have presented, the incidence of bronchiolitis obliterans syndrome which is diagnosed by the clinical criteria is lower, but it does not reach statistical significance. As you know, bronchiolitis obliterans syndrome, is diagnosed based on the reduction in the FEV₁. It reflects a global picture of the lung

function, and it may be more sensitive than the histological data from the transbronchial biopsy, in which sampling errors undoubtedly play a role.

The second question is about the future direction of this study. I presented these data as an interim report of a pilot study to assess the safety issue of this procedure. Any time one infuses unmodified bone marrow into a patient, especially in a lung transplant recipient who has a high number of passenger leukocytes, one is concerned about the development of graft-versus-host disease, which has been reported by the liver transplant group at the University of Miami. Therefore, we proceed very cautiously. We first show that it can be safely done, and then we escalate our conditioning regimen slowly.

Doctor Camillo Ricordi and associates at the University of Miami had shown a survival advantage in liver recipients who received multiple infusion of donor bone marrow versus those who received single infusion. Based on these data, we currently have initiated the multiple bone marrow infusion protocol in lung recipients.

Another approach is to escalate the conditioning regimen of the recipient. However, as one escalates the conditioning regimen, one increases the risk of graft-versus-host disease. Recently, Dr Suzanne Ildstad and her associates had demonstrated a population of cells, facilitating cells (FC), in the bone marrow that facilitate the engraftment of BM stem cells without causing graft-versus-host disease. By combining facilitating cells and bone marrow stem cells infusion, low dose total body irradiation, and conventional immunosuppression, one may achieve better bone marrow engraftment with minor risk of graft-versus-host disease. This protocol is being evaluated at several centers by Dr Ildstad and associates.

I am grateful for Dr Grover's kind comments. Patients who have chronic rejection lost their chimerism earlier than others. The major difficulty with this protocol was to obtain consent for donor bone marrow. In our organ procurement area, we were able to get bone marrow from 30% to 50% of cadaveric solid organ donors. Outside our area, the rate of getting donor bone marrow is less than 5%. I think with more public education, the rate of bone marrow donation in cadaveric donors will increase.

I would like to thank the Society for the opportunity to present this paper.