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## Brief Report

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# Flow Cytometric Evaluation of Red Blood Cell Chimerism after Bone Marrow Transplantation in Iranian Patients: A Preliminary Study

Mojgan Shaiegan PhD\*, Esmerdis Hadjati MSc\*\*, Mahnaz Aghaiipour MD\*\*\*,  
Masoud Irvani MD†, Gaelle David BS‡, Daniel Bernard BS‡

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The aim of this study was to evaluate mixed red cells population and red blood cell chimerism after hematopoietic stem cell transplantation. Red blood cell chimerism after hematopoietic stem cell transplantation was analyzed using a series of fluorescein isothiocyanate-conjugated monoclonal antibodies (BioAtlantic, France) directed against ABH, Rh (D, C, E, c, e), Kell, Duffy, Kidd, and Ss antigens on blood samples of 14 patients with hematologic disorders undergoing hematopoietic stem cell transplantation, by flow cytometric method on days 15, 30, and 60 after transplantation. All patients showed expression of donor red cell antigens within days 15 – 30 after hematopoietic stem cell transplantation. Graft versus host disease and ABO incompatibility did not affect the expression of chimerism.

Flow cytometric analysis is a simple, accurate, and valuable test which is of significant help in monitoring chimerism in allogeneic hematopoietic stem cell transplantation.

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## Introduction

Bone marrow transplantation (BMT) allows treatment of different diseases, including hematologic disorders, some anemias, and severe combined immunologic deficiencies (SCID).<sup>1</sup> The follow-up of patients after BMT is crucial in assessment of engraftment and in detection of mixed hematopoietic chimeras.<sup>2</sup> Detection of mixed red blood cell (RBC) population by agglutination test has been used for several years by Petz et al and van Dijk. However, the limit of the results is in the range of 3 – 5% of positive cells.<sup>1,2</sup> Flow cytometry is another method

which has been widely used in the detection of erythrocyte subpopulation. It was developed by David et al, Blanchard et al, and Hendriks et al,<sup>1-3</sup> and has a sensitivity of 0.1 – 0.3% as reported by Schaap et al<sup>4</sup> or 1% according to David et al.<sup>1</sup>

In the first step of our study, we tried to set up and use RBC phenotyping by flow cytometry for sequential analysis of chimerism in 14 patients with hematologic disorders before BMT, up to 60 days after transplantation. To continue our study, we are going to analyze RBC chimerism by flow cytometry over a longer period.

## Patients and Methods

Blood samples of 14 bone marrow transplanted patients were obtained from the BMT Center of Shariati Hospital, Tehran, Iran. These patients were suffering from hematologic disorders (6 had acute myelogenous leukemia [AML], 4 acute lymphocytic leukemia [ALL], 3 chronic myelogenous leukemia [CML], and 1 had myelodysplastic syndrome [MDS]). Ten (71.4%) patients were males and four (28.6%) were

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**Authors' affiliations:** \*Department of Immunology, \*\*Department of Flow Cytometry, \*\*\*Department of Immunology and Flow Cytometry, Iranian Blood Transfusion Organization Research Center (IBTORC), †Department of Hematology/Oncology, BMT Research Center, Shariati Hospital, Tehran, Iran, ‡Department of Biotechnology and Hematology, Center Regional de Transfusion Sanguine, Nantes, France.  
•**Corresponding author and reprints:** Mojgan Shaiegan PhD, Iranian Blood Transfusion Organization Research Center (IBTORC), Tehran, Iran.  
Fax: +98-21-8860-1555, E-mail: shaiegan@ibto.ir  
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females, aged between 10 and 42 years. Nine out of 14 patients were conditioned with fludarabine. All donors were human leukocyte antigen (HLA)-identical siblings.

Blood samples were collected in EDTA tubes for phenotyping by flow cytometry from donors and patients before BMT and from patients on days 15, 30, and 60 after transplantation. A blood sample with anticoagulant agent was also taken to collect sera for antibody screening, before and on day 60 after transplantation.

## Methods

Blood samples were centrifuged for 10 min at 600×g and the total RBCs were washed three times with phosphate-buffered saline (PBS) (pH 7.4). The cells were then suspended in PBS at a concentration of  $1 \times 10^9$  cells/mL, when it was necessary to transfuse red blood products to one of the patients. When we could not detect blood group antigens for all the transfused blood units, we just detected a blood sample of transfused units to determine the blood group antigens of transfused RBCs. It was also applied whenever there was a similarity between donor-recipient pair RBC antigens and transfused cells. We excluded patients if there was similarity between donor-recipient pair RBC antigens and transfused cells.

Flow cytometric analysis was performed using monoclonal antibodies against ABH, Rh antigens, and FY a, FY b, JK a, JK b, S, s, K, and k antigens conjugated with fluorescein isothiocyanate (FITC). They were purchased from BioAtlantic (Nantes, France). According to the manufacturer's instructions, for direct staining of most antibodies, 5  $\mu$ L of washed cells were incubated with 100  $\mu$ L of antibody for 30 min at room temperature and subsequently washed three times with PBS.

In case of indirect staining for A, B, and Rh antigens (D, C, c, E, and e), 5  $\mu$ L of washed cells were first incubated with 100  $\mu$ L of antibody for 60 min and subsequently washed three times with PBS and then 100  $\mu$ L of FITC-conjugate secondary antibody was added and incubated for 30 min at room temperature. It was then washed twice. The washed cells were resuspended in PBS.

Agglutination was not our problem, but if it occurred during centrifugation after sensitization with anti-A and anti-B antibodies, this problem was solved by introduction of overnight fixation stage (according to the manufacturer's instructions) prior to labeling with conjugated

antibodies and agglutinates were dissociated by repeated pipeting; then the RBCs were suspended by vortexing before analysis by fluorescence-activated cell sorter (FACS) (Partec PAS III, with a voltage of 601 MHz). Correct gating was assessed with a lineage-specific control, being antiglycophorin A+B. Over 99% of positive cells were detected.

In every run, we used negative and isotype controls and artificial mixtures (a mixture of 50% antigen-negative and 50% antigen-positive RBCs). Results were reported as the percentage of positive cells identified with the antibodies directed against blood antigens present on recipient RBCs or blood group antigens present on donor RBCs and absent on recipient RBCs.<sup>1-2</sup> Antibody screening was performed to detect antibodies in recipient's samples before and 60 days after HSCT.

## Results

### Occurrence of RBC chimerism

Our results showed that donor erythrocytes could be detected within 15 – 30 days after transplantation. The mean fluorescence intensity of negative controls in every run for different antigens was <3%.

### Graft-versus-host disease after BMT

Three patients developed acute graft-versus-host disease (GVHD) (grade I and II) on days 11, and 26 after HSCT; one developed chronic GVHD. However, they showed donor red cell chimerism on days 15 and 30 post-BMT, respectively.

### ABO incompatibility

There was ABO incompatibility in four recipient-donor pairs (shown in Table 1), but all showed donor red cell chimerism in their circulation up to day 30 after BMT.

We detected titer of anti-B and anti-A antibodies in recipients' sera before and after BMT, and found that the isohemagglutinin titer decreased after BMT in ABO incompatible pairs.

**Table 1.** ABO incompatibility between donors and recipients.

Donor blood group	Recipient blood group	Day of appearance of donor blood in recipient circulation
B	O	15
B	O	15
B	A	15
A	AB	30

ABO incompatibility did not affect the occurrence of donor red cell chimerism, just covering the donor blood antigens and made detection of chimerism difficult. But decreasing the percentage of H antigen in patient number 5 and other blood antigens in other patients, showed RBC chimerism based on flow cytometric results.

### Fludarabin regimen

Our results showed chimerism in patients who received fludarabine, appearing on day 15 after BMT. It seems that this drug does not delay the appearance of RBC chimerism in comparison to patients who received busulfan and cyclophosphamide.

## Discussion

In our study, nine (64%) patients showed chimerism on day 15 and five (36%) showed it on day 30 after HSCT. The frequency of females in comparison to males, among the patients who showed chimerism on day 15 posttransplantation was higher ( $P < 0.05$ ), but we suggest that the effect of sexuality on the occurrence of chimerism, should be evaluated with larger samples. Chimerism was detected in all patients, in spite of ABO incompatibilities. Antibody screening test confirmed that IgG and IgM antibodies against blood antigens (except in four ABO-incompatible patients) were not present in patients' sera and that isohemagglutinin titer decreased in ABO-incompatible patients after transplantation.

After allogeneic BMT, some patients still had residual recipient hematopoiesis. The co-existence of recipient's and donor's hematopoietic systems is called mixed chimerism. Some patients may remain in stable mixed chimerism, while others may convert to complete donor chimerism or graft rejection. The prediction of final outcome of mixed chimerism may help to decide interventions and prevent graft rejection. Flow cytometry has been found to be very useful for studying mixed cell populations. The other applications include to measurement of the amount of fetomaternal hemorrhage, determination of survival/clearance of transfused RBCs, phenotyping of transfused patients' RBCs, evaluation of BMT engraftment,<sup>6</sup> and study of RBC chimerism.<sup>5-7</sup> Engraftments could be demonstrated earlier by flow cytometry than agglutination, since only a minority of the population (1 – 10%) of cells could be determined

accurately with labeled reagents.<sup>2</sup> This method could help to detect early production of RBCs by engrafted stem cells and consequently help physicians to diagnose engraftment and decide the appropriate therapeutic strategy. From our limited series of patients, engraftment could be demonstrated around days 15 – 30.

Our results agreed with those reported by other researchers. Blanchard et al<sup>1</sup> reported expression of donor RBC antigens (expansion markers) and a concomitant decrease of recipient specific antigens (depletion markers) within days 16 – 20 in 125 successfully engrafted patients. In another study,<sup>2</sup> they reported that engraftment could be demonstrated around days 15 – 20 in most cases. Hendriks et al reported the earliest detection of donor erythrocytes 14 days after BMT.<sup>3</sup>

A simple dual-color flow cytometric method developed for antigen typing of reticulocytes in mixed red cell populations has been reported. This method provides a simpler, safer, less labor-intensive, and less subjective technique requiring far less sample volume than current methods for antigen typing of reticulocytes in mixed red cell samples from recently transfused patients.<sup>8</sup> van Dijk suggested that the persistence or reappearance of autologous erythrocytes in small percentages (0.05 – 10%) occurred without relapse of leukemia. Reappearance in high percentages (50 – 100%) indicated relapse.<sup>9</sup> Because of different limitation in our study, we did not follow up our patients for a long time. Hence, we could not obtain such results.

Since ABO and HLAs are inherited independently, ABO incompatibility may occur in up to 20 – 40% of HLA-matched allogeneic hematopoietic stem cell transplants (SCTs).<sup>10</sup> There was a report on ABO incompatibility in a BMT recipient demonstrating mixed RBC chimerism.<sup>11</sup> We report the same results in four recipients, demonstrating mixed RBC chimerism, without any apparent clinical complications due to ABO incompatibilities.

Bolan et al<sup>10</sup> have reported that delayed donor red cell engraftment and pure red cell aplasia (PRCA) are well-recognized complications of major ABO-incompatible hematopoietic SCT performed by means of myeloablative conditioning. To evaluate these events following reduced-intensity nonmyeloablative SCT (NST), consecutive series of patients with major ABO incompatibility undergoing either NST

(fludarabine/cyclophosphamide conditioning) or myeloablative SCT (cyclophosphamide/high-dose total body irradiation) were compared. Donor RBC chimerism (initial detection of donor RBCs in peripheral blood) was markedly delayed following NST versus myeloablative SCT (median: 114 vs. 40 days;  $P < 0.0001$ ) and strongly correlated with decreasing host antidonor isohemagglutinin levels. The onset of donor red cell chimerism was delayed over 100 days in 9 of 14 patients following major ABO-incompatible NST, with four of these patients developing pure red cell aplasia. In contrast, there were no patients with delayed donor RBC chimerism or PRCA in the 12 concurrent cases of major ABO-incompatible myeloablative SCT. We showed that fludarabine regimen and ABO incompatibility did not affect the appearance of chimerism.

We conclude that flow cytometric analysis is a simple and sensitive test and a valuable method, which could be a significant help in monitoring chimerism in allogeneic BMT. It may help to detect recipient RBCs, which is indicative of possible relapse of the initial disease. In addition, analysis of reticulocytes from reticulocyte-rich fraction allows early identification of the cells originating from the transplanted progenitor cells.

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