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Erythroid cells

Erythrocyte antigen and reticulocyte engraftment after allogeneic hematopoietic stem cell transplantation

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Summary:

The aim of this study was to study the usefulness of erythrocyte antigen (EA) measurement to study engraftment after allogeneic HSCT. In all, 31 consecutive patients receiving HLA-identical bone marrow (BM) ($n = 13$) or peripheral blood stem cells ($n = 18$) were investigated. Apart from the ABO group, 15 EAs representing six minor blood groups were followed by the simple tube agglutination technique. A total of 20 (64.5%) patients received ABO-identical, eight (25.8%) received ABO minor and three (9.7%) received ABO major mismatched grafts. In all, 29 patients were followed for a median of 12 (6–16) months; 65% of the patients expressed donor type EA 1 month and almost all did so 6 months after transplant. Reticulocyte engraftment was significantly shorter than EA engraftment (median 18 *vs* 35 days) ($P = 0.001$). Patients who received PB stem cells showed significantly faster EA and reticulocyte engraftment than patients who received BM stem cells ($P = 0.038$ and 0.025). ABO compatibility did not have an impact on reticulocyte and EA engraftment ($P = 0.4$ and 0.55). The earliest donor type EA detected was from the Rh and Kidd system. These data suggest that EA and reticulocyte assays are useful in monitoring engraftment.
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Cytogenetics and molecular methods are commonly used to analyze allogeneic engraftment, but the high cost and the need for skilled staff are disadvantages. The difference between erythrocyte antigens (EAs) of donor and patient origin as evidence of engraftment and chimerism has been studied since the 1980s.[1,2] The most common method used is RBC agglutination. Later, anti-human IgG-coated fluorescence microspheres were used to detect chimerism.[3] Subsequently, gel test was used to differentiate donor and recipient RBCs.[4] Flow cytometric analysis using monoclonal antibodies directed against blood group antigens enabled sensitive evaluation of dual RBC populations.[5]

Our aim was to study a simple tube hemagglutination method for monitoring RBC repopulation following HSCT. In addition, we evaluated the disappearance of recipient-derived RBC antigens and appearance of donor-derived antigens after transplantation, and correlated RBC antigen engraftment with reticulocyte counts after AHSCT.

Patients and methods

Patients

A total of 31 consecutive HSCT patients were studied. They consisted of 11 females and 20 males with a median age of 36 years (range 18–51). The underlying diseases were chronic myeloid leukemia in 18, acute leukemia in 11, severe aplastic anemia in one and renal cell carcinoma in one patient. In all, 13 of the patients received bone marrow (BM) and 18 received peripheral blood stem cells (PBSC) from their HLA-identical siblings. Eight of the patients received minor and three received major ABO-incompatible grafts. Table 1 shows the patient and donor characteristics.

Transplantation procedure

The patients were conditioned by ablative cyclophosphamide-based regimens (81%) or fludarabine-based reduced intensity regimens.[6] All the patients received short-course methotrexate and cyclosporine for GVHD prophylaxis. All the donor–recipient pairs were CMV-seropositive. Recombinant human G-CSF was used until neutrophil engraftment after day +1 in BM group, but not in the PBSC. All the blood products were leucofiltered and irradiated. The pretransplant evaluation of the mismatched patient–donor pairs, the decision for erythrocyte and plasma depletion of the harvest and post-transplant transfusion policy were designed by our transplant center's transfusion medicine consultant (Dr Arslan), according to recently published guidelines.[7]

Blood group typing

Apart from the ABO group, 15 EA (C, c, E, e, Fy^a, Fy^b, Jk^a, Jk^b, K, k, M, N, S, s and P_1) representing six minor blood groups were followed by the simple tube

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agglutination technique in our blood bank. The results were evaluated from 1+ to 4+ according to the intensity of agglutination. Erythrocyte antigens were determined by using 15 murine monoclonal antibodies: anti-C, anti-c, anti-E, anti-e, anti-K, anti-k, anti-S, anti-s, anti-Jka, anti-Jkb, anti-Fyb, anti-M, anti-N, and anti-P1 (Dominion, Canada). Positive and negative controls were used for each antigen. For positive controls, cells expressing heterozygous antigen for the erythrocyte antigen were selected. The C, c, E and e antigens were evaluated at 37°C and M, N and P1 antigens were evaluated at room temperature. K, k, S, s, Jka, Jkb, Fya and Fyb antigens were examined in AHG (Anti Human Globulin) phase and anti-Ig murine monoclonal blend Coombs' reagent was used (Dominion, Canada).

All these antigens were typed for the donor and patient before the transplant and the different antigens that the patient carries but not the donor or *vice versa* were followed for erythrocyte engraftment (Table 2). All the laboratory work-up was performed by two experienced laboratory technicians.

RBC matching

RBC transfusions during the peri and post-transplant period were carefully selected not to mix up the antigens followed. Patients who received blood transfusions for the last 3 months before the transplants were not eligible for the study. The 'Hemosoft' blood bank software was used to find a matched RBC unit in the pool. One patient was excluded from the study because of violation of the study protocol's transfusion policy.

Table 1 Patient and donor characteristics according to blood group matching

	ABO matched		*ABO minor mismatched*		*ABO major mismatched*	
	BM	*PBSC*	*BM*	*PBSC*	*BM*	*PBSC*
N	11	9	1	7	1	2
Sex						
Matched	6	3	1	5	1	1
Mismatched	5	6	—	2	—	1
Median age (range)	33 (18–46)	36 (25–46)	38	30 (26–34)	26	31,48
Diagnosis						
AL	2	5	0	3	0	1
CML	8	3	1	4	1	1
Others	1	1	—	0	—	—
Conditioning regimen						
Cy based	10	6	1	6	1	2
Reduced intensity	1	3	—	1	—	—
Blood groups (donor/patient)	O/O = 6 A/A = 5	O/O = 2 A/A = 5 B/B = 2	O/A = 1	O/A = 5 O/B = 1 B/AB = 1	A/O = 1	AB/A = 1 AB/B = 1

BM = bone marrow, PBSC = peripheral blood stem cell, Cy = cyclophosphamide, AL = acute leukemia, CML = chronic myelogenous leukemia.

Table 2 Disparity of EA between donor and recipient

Patient no.	*Donor*	*Recipient*	*Patient no.*	*Donor*	*Recipient*
1	K	Jk^b	17	Fy^a, s	C
2	—	K	18	Jk^b	C, Fy^b
3	S	—	19	E	—
4	E	N	20	Jk^b	c, E, P_1, S
5	Fy^a	P_1	21	C, E	—
6	Jk^a, Jk^b	c, E, P_1	22	c	Jk^b
7	S	Jk^a	23	C, Fy^b	—
8	C, E	Fy^a, N	24	s	—
9	—	c, Fy^a, Jk^b	25	Jk^b	C
10	s	Jk^b	26	P_1	C
11	—	Jk^b, s	27	C	Fy^b
12	e	—	28	C	—
13	Fy^b	—	29	C, M	—
14	Fy^a	Fy^b, P_1, S	30	c, M	K, N, P_1
15	S	Jk^a, P_1, s	31	Jk^a	K, Fy^a
16	S	Jk^a			

Follow-up

After transplantation, patients were followed up for 45 days, with blood sampling weekly to analyze the blood group antigens. Patients who were discharged from the hospital were followed for the EA at the 1st, 2nd, 3rd and 6th months. Coombs' tests (DAT) of the ABO compatible and incompatible transplants were studied once a month and thrice weekly, respectively. The titers of anti-A, anti-B and the levels of LDH (normal range 100–190 IU/l) and bilirubin (normal range 0.1–1.2 mg/dl) were followed thrice weekly during the hospitalization period and at routine check-up after discharge in ABO incompatible transplants. Haptoglobin levels were measured in case of clinical suspicion of hemolysis.

Definition of engraftment

Peripheral blood reticulocyte, WBC and platelet counts were monitored by automated hematology analyzers Gen S and STKS (Beckman Coulter, Miami, FL, USA). Engraftment was defined as the first day of reticulocyte count $>1\%$, granulocyte count $>0.5\times10^9$/l and an unsupported platelet count $>20\times10^9$/l for three consecutive days. The first day of appearance of donor type EA was accepted as EA engraftment.

Definition of immune hemolysis

Immune hemolysis was defined as a decrease in Hb level of more than 2 g/dl, decrease of haptoglobin levels within 24 h, increase of LDH of more than 50% above the previous day's value, total serum bilirubin levels of at least two times the normal value, and a positive DAT.

Statistical analysis

Ordinal data are expressed as medians and ranges. Statistical analyses were performed by using a commercial statistical software package. Student's *t*-test was used to compare the reticulocyte, granulocyte and platelet engraftment according to stem cell source. Other analyses were done by using the χ^2 test. *P*-value <0.05 was accepted as statistically significant.

Results

Two of the 31 patients were excluded from the study; one of them received EA-incompatible transfusion, and the other developed early relapse. All the patients were transplanted successfully and no early transplant-related mortality was seen in this cohort of patients. The remaining 29 patients were followed in a median of 12 (range 6–16) months.

Engraftment of the erythrocyte antigens

Donor-derived EAs were detected within a median of 35 (28–70) days after transplantation. In 65% of the patients donor-derived EAs were positive on the first month. All EAs were donor derived 6 months after transplantation. The recipient type EA disappeared 80 (21–130) days after the procedure. The time for the appearance of donor-derived antigens was significantly shorter than the time for the disappearance of recipient antigens ($P=0.0001$). The median time (days) of disappearance of recipient's EA and appearance of donor EA for K, Fya, Fyb, Jka and Jkb were 38–42, 40–46, 78–33, 56–28 and 34–28, respectively. The median times for the disappearance of recipient EA and appearance of donor EA for the remaining subgroups were 105 and 25 days, respectively.

The median days of disappearance of recipient's EA for Kell, Duffy, Kidd, MNSs, P and Rh antigens were 38, 60, 45, 90, 75 and 96, respectively, whereas these periods for the appearance of donor type EA for the same group were 42, 40, 28, 44, 28 and 24 days.

There was no difference for the appearance of donor EA in ABO compatible and incompatible transplants (31 *vs* 24 days) ($P=0.55$) (Figure 1). There was also no difference for the disappearance of EA between the two groups ($P=0.9$). Owing to a small number of patients, no analyses were carried out for the appearance and disappearance of EA within the ABO mismatched group (minor *vs* major) and conditioning regimen (ablative *vs* reduced intensity). However, the median times for the appearance of antigens in major and minor mismatched groups were 33 and 24 days, respectively.

ABO blood group change and transfusion requirements

Donor type ABO blood group switch was observed in a median of 90 (range 45–160) days after transplant. In ABO major mismatched patients, this switch was noticed on day 57 (range 45–73). We were not able to show any statistical difference in terms of blood group switch between minor (median 96 days; range 45–160) and major mismatched transplants ($P=0.17$). The RBC transfusions in the first 30 days for the ABO compatible and incompatible transplants were 2.5 units (range 0–9) and 2.5 units (range 3–10) respectively. There was also no difference between the two groups 100 days after transplantation (4.7 units *vs* 6 units) ($P=0.9$). No statistical difference was encountered for the RBC transfusion need for PBSC and BMT patients (2.25 *vs*

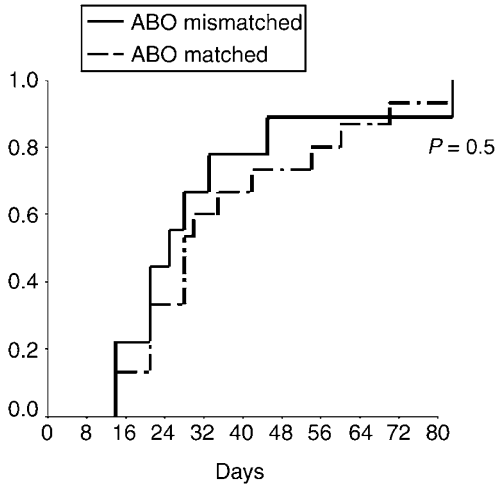


Figure 1 Erythrocyte antigen engraftment in ABO matched *vs* mismatched patients.

3.2 units) ($P=0.27$). The median random platelet transfusion requirement for the ABO compatible and incompatible groups was 17.1 units (range 0–53) and 18.6 units (range 0–48), respectively ($P=0.75$). The platelet transfusion requirements in the PBSC transplant group were statistically less than the BMT group (13 *vs* 23) ($P=0.04$).

Reticulocyte engraftment

A total of 28 patients were followed up for reticulocyte engraftment. The median time for engraftment was 18 days (range 12–50) for all patients. Reticulocyte engraftment was significantly shorter than EA engraftment ($P=0.001$) (Figure 2). Three patients showed concordant reticulocyte and EA engraftment on the same day (+14, +14, +28). The engraftment for reticulocytes and EA was earlier in patients undergoing PBSCT rather than BMT (Table 3). No difference was encountered between ABO compatible and incompatible groups for reticulocyte engraftment.

Granulocyte and platelet engraftment

Although the granulocyte engraftment was faster in the PBSCT group than the BMT group (15 *vs* 17 days), no statistical difference was found ($P=0.1$). The median time for granulocyte engraftment was 16 (range 7–26) days for all patients. Also, we could not demonstrate any difference between ABO compatible and incompatible groups ($P=0.9$). The median time for platelet engraftment was 17 days (range 10–30) for the whole group. The time for platelet engraftment was significantly shorter in PBSCT than in BMT (14 *vs* 21 days, respectively; $P=0.001$). There was no statistical difference between the ABO compatible and incompatible groups in terms of platelet engraftment ($P=0.14$).

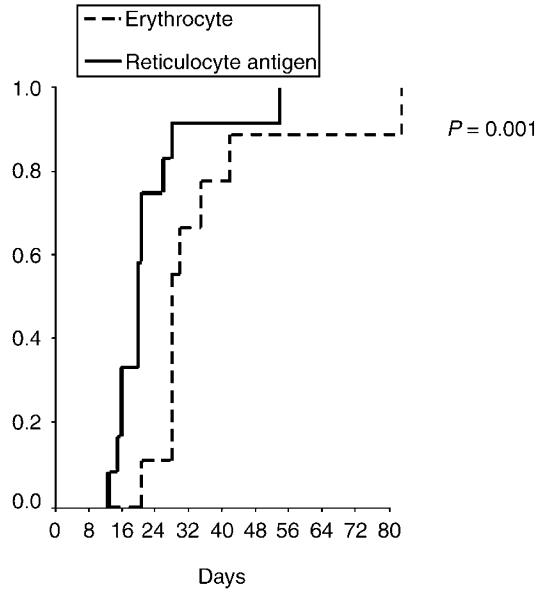


Figure 2 Comparison of erythrocyte antigen and reticulocyte engraftment for the whole group.

Early and late hemolytic complications

We did not observe any early hemolytic complication in either major or minor ABO mismatched transplants. Only one patient developed a late hemolytic reaction (3.4%) on day +28. A raise in the level of unconjugated bilirubin (from 0.5 to 3.9 mg/dl) and LDH (from 231 to 379 U/L) was encountered in this major ABO mismatched patient, who was O Rh+ and whose donor was A Rh+. The patient was C (−), Fy^{b} (+) and the donor was C (+), Fy^{b} (−). There was a sharp increase of anti-A titer (1/1024) on day +32.

Discussion

The composition of RBC antigens is important in transfusion therapy. In humans 24 blood group systems have been well defined and there are still unknown antigens and new blood group systems to be defined. The most important groups that can cause severe hemolytic reactions and are highly immunogenic are ABO and Rh systems. The other RBC antigens studied normally feature in investigations of the inheritance of genes for forensic medicine.

To evaluate the chimerism after bone marrow or peripheral blood stem cell transplantation, various methods such as EA, immunoglobulin allotypes, granulocyte antigens, erythrocyte and leukocyte enzymes, DNA RFLP and PCR are used.[8–12] The sensitivity of cytogenetic analysis to demonstrate the minor cell populations is limited by the cell count on the metaphase plate. Sometimes it may be difficult to obtain adequate number of cells after transplantation. RFLP is a highly sensitive method; however, there is a need for qualified staff and specialized equipment and it is time consuming.[9,13,14] Using EA to demonstrate chimerism provides an opportunity to follow the process of engraftment using red cells alone,[15–17] and offers the advantages of simplicity and speed without requirement for either special equipment or staff.

Various methods have been used to demonstrate the EA. De Man *et al*[3] studied the erythrocyte repopulation using antibody-coated fluorescent microspheres in 22 patients

Table 3 Analysis of median time to erythrocyte antigen and reticulocyte engraftment according to stem cell source

Time (days)	*Allo-BMT (n = 11)*	*Allo-PBSCT (n = 18)*	*P*	*All patients (n = 29)*
Reticulocyte engraftment (range)	22 (13–28)	16 (12–21)	0.025	18 (12–50)
EA engraftment (range)	37 (21–83)	29 (14–70)	0.038	35 (28–70)
EA disappearance (range)	69 (14–111)	75 (21–110)	0.67	80 (21–130)

EA = erythrocyte antigen, BMT = bone marrow transplantation, PBSCT = peripheral blood stem cell transplantation.

who received bone marrow transplantation, achieving success in 19. In 1993, gel test was used to evaluate the mixed erythrocyte repopulations of 38 allogeneic bone marrow transplant patients.[4] Soon after, erythrocyte engraftment was analyzed by using flow-cytometric methods.[18–20] The tube agglutination method used in our study is not a commonly used method in the literature. There are only a few studies regarding the chimerism of EA and most of them consist of a small number of different antigens. In our study, 15 different antigens between the donor and the patient were followed up and engraftment was recorded. Donor EA became positive in 65% of our patients in the first month following transplantation. In a study carried out by using antibody-coated fluorescent microspheres, donor-derived EA was seen in 86% of the patients on the 15th day.[3] In another study, donor-derived antigens were observed at the end of 8th week in 71% of patients.[1] It is concluded that the tube agglutination technique is as efficient as antibody-coated fluorescent microspheres or gel tests in order to detect EA engraftment. In our study, donor-derived EA became 100% positive on the sixth month and during this period recipient erythrocyte antigens mostly disappeared.

The blood group systems where changes could first be detected were in the Rh and Kidd systems, and the first to become undetectable was in the Kell system. There are no available data about this issue in the literature. The early appearing Rh and Kidd antigen systems may be used as a parameter to demonstrate early chimerism.

Some authors underlined that the first determinant of relapse was the progressive decline of the present donor-derived erythrocyte antigens and disappearance or the initial absence of donor erythrocytes.[1,2] Van Dijk *et al*[1] reported that the appearance of autologous EA was a sign for relapse of underlying disease. Bar *et al*[21] showed the persistence of autologous EA in patients who had relapsed. Similarly, we were not able to demonstrate the donor-derived EA in two of our patients who subsequently relapsed.

Our data suggest that evaluating erythrocyte repopulation by the tube agglutination method is feasible and cost effective in patients who received allograft from ABO mismatched HLA-identical sibling donors. After transplantation, erythrocyte antigens can be used to demonstrate chimerism.

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