Significance of Donor-Derived Isoagglutinins in ABO-Incompatible Hematopoietic Stem Cell Transplantation

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Changes in isoagglutinin titers may have implications in the occurrence of hematological complications such as pure red cell aplasia or immune-mediated hemolysis. Furthermore, isoagglutinin titers could reflect immunohematological reconstitution after transplantation. The objective of this study was to examine the relationship between donor-derived isoagglutinins (DDIs) and graft-versus-host disease (GVHD). In total, 114 patients who underwent ABO-incompatible allogeneic hematopoietic stem cell transplantation (HSCT) were analyzed. Among these patients, 27.7% demonstrated increased donor-derived isoagglutinins (IDDI) against red blood cells (RBCs) of the recipient, and 32.8% of the patients showed DDIs that were not against RBCs of the recipient.

Patients with acute GVHD and DDIs against RBCs of the recipient tended to have higher incidences of IDDI that occurred before posttransplant day 60 compared with patients without acute GVHD (17.3 vs. 3.9%, $P=0.058$). In patients with acute GVHD, IDDI occurred significantly earlier (mean, day 32 vs. 181, $P=0.046$), the period of elevation was shorter (mean, day 36 vs. 134, $P=0.033$), and the donors were younger (mean, 28 vs. 36 years, $P=0.01$) than those without GVHD. Moreover, significant correlations were found between IDDI and acute GVHD. Taken together, these data underscore a possible role for humoral immunity in GVHD after HSCT. *J. Clin. Lab. Anal.* 22:383–390, 2008. © 2008 Wiley-Liss, Inc.

Key words: donor-derived isoagglutinin (DDI); graft-versus-host disease (GVHD); ABO incompatibility; hematopoietic stem cell transplantation (HSCT)

INTRODUCTION

ABO mismatch in hematopoietic stem cell transplantation (HSCT) can lead to several clinical problems (1,2) such as immediate hemolysis (3), delayed hemolysis (4,5), delayed erythroid engraftment including pure red cell aplasia (PRCA) (5,6), an increased need for transfusion of neutrophils or platelets (7), and increased graft-versus-host disease (GVHD) (8,9). Specifically, GVHD remains a major cause of morbidity and mortality following allogeneic HSCT. Many studies have shown that CD4⁺ T cells play an important role in the pathogenesis of GVHD, and T-cell depletion of donor grafts is considered as an effective method of preventing GVHD. Although previous studies have not demonstrated a decisive role for B lymphocytes in the development of GVHD, we postulated that the function of B lymphocytes during immunohematological recon-

stitution after allogeneic HSCT may instigate GVHD. Regular follow-up analyses of isoagglutinin titers can be used to monitor erythroid engraftment and to detect development of any complications after ABO-incompatible allogeneic HSCT (8,10). Previously, we showed that changes in isoagglutinin titers might have clinical

Abbreviations: DDI, donor-derived isoagglutinin; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; IDDI, increased donor-derived isoagglutinin; PRCA, pure red cell aplasia; RDI, recipient-derived isoagglutinin.

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TABLE 1. Changes in Isoagglutinins in ABO-Incompatible Hematopoietic Stem Cell Transplantation

ABO incompatibility	Recipient	Donor	RDI	→	DDI
Major	A	AB	Anti-B		—
	B	AB	Anti-A		—
	O	AB	Anti-A, anti-B		—
	O	A	Anti-A, anti-B		
	O	B	Anti-A, anti-B		
Minor	A	O	Anti-B		Anti-A
	B	O	Anti-A		Anti-B
	AB	O	—		Anti-A, anti-B
	AB	A	—		Anti-B
	AB	B	—		Anti-A
Major and minor	A	B	Anti-B		Anti-A
	B	A	Anti-A		Anti-B

Objectives of this study are boxed in dotted and solid lines. RDI, recipient-derived isoagglutinins; DDI, donor-derived isoagglutinins.

relevance and could promote immunohematological complications such as PRCA or immune-mediated hemolysis. Moreover, isoagglutinin titer changes might reflect immunohematological reconstitution after transplantation; the decrease in recipient-derived isoagglutinin (RDI) appears significantly earlier in the presence of acute GVHD owing to the graft-versus-plasma cell effect (11).

After HSCT, major incompatibility may be altered because of the RDI to donor-derived isoagglutinin (DDI) change. However, the point at which major incompatibility is altered is unclear and the changing patterns themselves are diverse (10,11). Loss of RDI appears to be related to the engraftment of hematopoietic cells. This is different for DDI because DDIs react with ABO antigens expressed on the endothelium. Moreover, detecting only DDIs is difficult in the presence of RDIs. We focused on the observation that increased donor-derived isoagglutinin (IDDI) augmented the activity of donor B lymphocytes. Concordantly, recent studies have shown that these B lymphocytes may play a role in GVHD (12,13). In this study, we investigated IDDI and its clinical association with acute or chronic GVHD in patients who underwent ABO-incompatible allogeneic HSCT (Table 1).

MATERIALS AND METHODS

Patients

A total of 132 adult patients underwent ABO-incompatible allogeneic HSCT at Asan Medical Center between January 1996 and June 2005. Patients were excluded if the follow-up period was less than 2 weeks or if isoagglutinin titers were investigated less than three times. The analysis was performed on 114 of the 132 patients and excluded AB-type donors. The subjects

could be divided into two categories (Fig. 1). The first category included patients with DDI against red blood cells (RBCs) of the recipient and the second category included patients with DDI that were not against RBCs of the recipient.

HSCT Procedure

The HSCT procedure was performed as described previously (11), and conditioning regimens were selected according to the underlying disease. Conditioning regimens were as follows: BuCy [$n = 83$; busulfan (Bu) 4 mg/kg/d for 4 days and cyclophosphamide (Cy) 60 mg/kg/d for 2 days] for leukemia/myelodysplastic syndrome, Cy-ATG [$n = 13$; Cy 50 mg/kg/d for 4 days and antithymocyte globulin (ATG) 30 mg/kg/d for 3 days] for severe aplastic anemia, and Bu-Fludara-ATG ($n = 18$; Bu 4 mg/kg/d for 2 days, fludarabine 30 mg/m²/d for 6 days, and ATG 10 mg/kg/d for 4 days) for nonmyeloablative conditioning. All patients received cyclosporine 1 day before the conditioning regimen for the prophylaxis of GVHD. In addition, 87 patients received methotrexate on days 1, 3, 6, and 11. To prevent hepatic venoocclusive disease, heparin was administered to those patients who received the BuCy regimen. In the cases of major (and minor) ABO incompatibility, the donor marrow was depleted of RBCs using the Baxter CS-3000 (Deerfield, IL) collection device before infusion. Recipients' isoagglutinins were not reduced before transplantation. In the cases of minor ABO incompatibility, plasma depletion of donor marrow was performed before graft infusion. Stem cell grafts were derived from bone marrow in 94 patients and peripheral blood in 20 patients. Peripheral blood stem cells were collected over 2 days by leukapheresis after mobilization with granulocyte colony-stimulating factor (10 µg/kg/d) for 4 days.

Fig. 1. Schematic presentation of patient groupings used in the analysis. Objectives of this study are boxed in dotted and solid lines.

Laboratory Evaluation

Blood (ABO) type and isoagglutinin titers were determined every week beginning 7 days prior to HSCT. For major (and minor) ABO incompatibility, titers were monitored until the isoagglutinins against the donor RBCs disappeared and donor-type RBCs appeared. For minor (and major) ABO incompatibility, titers were determined until the recipient RBCs disappeared. Anti-A and anti-B isoagglutinin titers were determined by incubating 0.1 ml of type A and B RBC suspensions in 0.1 ml of saline containing 2-fold serial dilutions of patient serum at room temperature followed by centrifugation. The agglutinating activity of IgM isoagglutinin was confirmed by treating the patient serum with 0.01 mmol/l dithiothreitol, which abolished its agglutinating property. Titers were scored for agglutination. Complete blood counts were determined daily until hospital discharge and were subsequently measured once a week at each outpatient visit. Bone marrow biopsy and aspiration were performed when neutrophil engraftment was established.

Increase of DDI Titers and GVHD

The IDDI was defined as the first appearance of DDI in patients with DDI against RBCs of the recipient or a

4-fold increase of DDI in patients with DDI not against RBCs of the recipient. We investigated the frequency and the patterns of IDDI and analyzed correlations between IDDI and the occurrence of GVHD on posttransplant days 30, 60, and 90. Factors affecting the occurrence of GVHD in patients with IDDI were also analyzed.

Statistical Analysis

The kinetics of isoagglutinin titers were calculated using the Kaplan–Meier method (14) and compared by log-rank analysis. Differences in categorical variables were tested using the χ^2 and Fisher's exact test. To determine how the variables were related, Pearson's correlation coefficients were calculated using the bivariate correlation procedure. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Patient Characteristics

Among the 312 adult patients who underwent allogeneic HSCT at Asan Medical Center, 145 (46.5%) received ABO-incompatible stem cell grafts. Adequate data regarding changes in isoagglutinin titers were

available for 132 patients. In total, 45 patients (34.1%) had major ABO incompatibility, 55 (41.7%) showed minor ABO incompatibility, and 32 (24.2%) had both major and minor ABO incompatibility. The clinical characteristics of 114 patients excluding the AB-type donors are given in Table 2. Median age was 33 years and ranged from 15 to 57 years. The mean follow-up duration was 245 days and ranged from 15 to 1,592 days.

Increase in DDI Against RBCs of the Recipient

Forty-seven patients had isoagglutinins against RBCs of the recipient. Increases in DDI against the RBCs of

TABLE 2. Characteristics of Patients in the Study ($n = 114$)

Characteristics	No. of patients (%)
Gender of recipient	
Male	63 (55.3)
Female	51 (44.7)
Gender of donor	
Male	75 (65.8)
Female	39 (34.2)
Types of stem cell donor	
Sibling	74 (64.9)
Unrelated	40 (35.1)
Diagnosis	
Acute myeloid leukemia	34 (29.8)
Acute lymphoblastic leukemia	22 (19.3)
Biphenotypic acute leukemia	2 (1.8)
Chronic myeloid leukemia	20 (17.5)
Myelodysplastic syndrome	13 (11.4)
Severe aplastic leukemia	13 (11.4)
Paroxysmal nocturnal hemoglobinuria	2 (1.8)
Others	8 (7.0)
ABO incompatibility	
Major	27 (23.7)
Minor	55 (48.2)
Major and minor	32 (28.1)
Total	114

the recipient were detectable in 27.7% of patients (13/47) who received minor (and major) ABO-incompatible stem cell grafts. The phenomenon of a posttransplant increase in isoagglutinin titers occurred at posttransplant day 21 (median; range, days 8–148). The titers gradually decreased with time and on an average disappeared by day 23. In two patients, however, the isoagglutinins persisted until the last date of observation (days 7 and 75 from the day of appearance). The maximal titers ranged from 1:2 to 1:128. The distribution was as follows: 1:2 in four patients; 1:8 in five patients; 1:16 and 1:64 in one patient each; and 1:128 in two patients. No significant correlations were observed between IDDI and other factors such as gender, age, type of isoagglutinin (anti-A or anti-B), donors of hemopoietic stem cells, acute GVHD, or chronic GVHD (data not shown). However, IDDI was more frequent in female than male recipients, although this trend did not reach statistical significance (23.08% female vs. 8.33% male, $P = 0.06$; Fig. 2B).

Increases in DDI That Were Not Against RBCs of the Recipient

In total, 67 patients had isoagglutinins that were not against RBCs of the recipient. In these patients, the titers decreased continuously in 11 patients, decreased but with transient increase in 23 patients, transiently increased followed by a decrease in 12 patients, and fluctuated in the remaining 21 patients. With exception to the former 11 patients, 56 patients (83.6%) showed increases in DDI during the study. Increases in DDI were observed in 32.8% (22/67) of the patients with DDI not against RBCs of the recipient. This posttransplant phenomenon was characterized by an increase in isoagglutinin titers that occurred at posttransplant day 47 (median). The titer increase ranged from days 8 to 793 in the patients. The titers gradually decreased with time and disappeared on an average by day 122. In six

Fig. 2. Appearance of donor-derived isoagglutinins against RBCs of the recipient in minor (and major) ABO incompatibility (group 1): (A) overall appearance, (B) frequency of appearance; female 23.08%, male 8.33% ($P = 0.06$). RBCs, red blood cells.

Fig. 3. Dual box-and-whisker plot showing (A) the appearance of IDDI according to the presence of acute GVHD, (B) the duration of IDDI according to the presence of acute GVHD, and (C) the presence of acute GVHD. IDDI, increased donor-derived isoagglutinin; GVHD, graft-versus-host disease.

TABLE 3. Relationship of IDDI With Acute or Chronic GVHD

IDDI	Acute GVHD			Chronic GVHD		
	Yes	No	<i>P</i> -value*	Yes	No	<i>P</i> -value*
Mean time to the appearance of DDI (d)	32	180.5	0.046	92.5	182.5	0.305
Mean interval of the persistence of IDDI (d)	36.1	133.8	0.033	88.7	129.6	0.447
Mean titer of DDI	84:1	46:1	0.358	62:1	61:1	0.986
Highest titer of DDI	512:1 (2 ⁹)	128:1 (2 ⁷)	0.328	512:1 (2 ⁹)	256:1 (2 ⁸)	0.659
Amount of increased titer						
2-Fold	3	11	0.245	5	7	1.000
3-Fold and greater	7	8		5	7	

**P*-value; *t*-test in comparing means and χ^2 test in comparing categorical data. DDI, donor-derived isoagglutinin; IDDI, increased donor-derived isoagglutinins; GVHD, graft-versus-host disease.

patients, the isoagglutinins persisted until the last date of observation (days 8, 81, 102, 169, and 235 from the day of appearance). The level of titer increase ranged from 4- to 32-fold. Specifically, 14 patients had a 4-fold increase in isoagglutinins, 6 patients demonstrated an 8-fold increase, and 1 patient each had a 16- and 32-fold increase in isoagglutinin. No significant correlations were observed between IDDI and other factors such as gender, age, type of isoagglutinin (anti-A or anti-B), donors of hemopoietic stem cells, acute GVHD, or chronic GVHD (data not shown).

Increase in DDI and GVHD

Correlation analysis of IDDI and GVHD on post-transplant days 30, 60, and 90 showed that patients with DDIs against RBCs of the recipient who also had acute GVHD frequently displayed IDDI prior to posttransplant day 60 compared with the subject group, which did not have GVHD (17.3 vs. 3.9%, *P* = 0.058). No

significant correlations were observed between chronic GVHD and IDDI after posttransplant day 60 in any of the patients (data not shown).

Relationship Between Acute or Chronic GVHD and IDDI

In the cases in which IDDI was observed, IDDI occurred significantly earlier, the period of elevation was shorter (mean, day 36 vs. 134, *P* = 0.033; Fig. 3), and the donors were younger (mean, 27.5 vs. 35.9 years, *P* = 0.01; Fig. 3) in patients with acute GVHD (mean, day 32 vs. 181, *P* = 0.046) compared with those without GVHD (Table 3). Other factors such as the recipient's gender or age, the donor's gender, and the types of donor revealed no significant differences in association with GVHD. Chronic GVHD was more frequent in female donors than in male donors (75 vs. 25%, respectively, *P* = 0.032); other factors were not different (Table 4).

TABLE 4. Characteristics of Patients With IDDI Who Developed Acute or Chronic GVHD

Variables	Acute GVHD			Chronic GVHD		
	Yes	No	<i>P</i> -value*	Yes	No	<i>P</i> -value*
Mean age of patient (yr)	28	34.5	0.08	29	34.14	0.159
Mean age of donor (yr)	27.5	35.9	0.01	31.6	34.2	0.494
Patient gender (<i>n</i>)						
Female	6	11	1.000	4	9	0.408
Male	4	8		6	5	
Donor gender (<i>n</i>)						
Female	2	7	0.431	6	2	0.032
Male	8	12		4	12	
Type of stem cell donor (<i>n</i>)						
Sibling	6	16	0.193	6	13	0.122
Unrelated	4	3		4	1	

**P*-value; *t*-test in comparing means and χ^2 test in comparing categorical data. *P* < 0.5 is expressed in bold. For abbreviations, see Table 3.

DISCUSSION

In this study, IDDI were analyzed after HSCT to evaluate factors, such as GVHD, that might promote immunological reactions in donors. In our previous study, patients with IDDI who demonstrated DDI against RBCs of the recipient accounted for 13.9% of the patients (5/36) (11). This study included a greater number of patients, and a similar proportion of patients were observed to be in this category as well. However, 82.8% of patients with IDDI (24/29) had DDIs that were not against RBCs of the recipient, which is remarkably different from that found in the previous study (11). In that study, all patients with IDDI showed a 2-fold increase in titer. Using this level of titer increase as a definition of IDDI indicates that in this study, 83.6% (56/67) of patients had IDDI.

Titer increases are influenced by various factors that may affect the activity of immunoglobulins such as antilymphocyte globulin, the origin of hematopoietic stem cells, and donor lymphocyte infusions. Moreover, these increases in titer may amplify immunological reactions caused by donor lymphocytes. In this study, an increase in the titer of 4-fold or greater was used as a strict definition of IDDI to exclude factors other than immunological reactions caused by lymphocytes from the donor. This approach is more reasonable because DDIs in patients with DDIs against RBCs of the recipient were detected in vivo after saturation of the ABO antigen on the endothelium. Because isoagglutinin in patients possessing DDIs that were not against RBCs of the recipient fails to react with erythrocytes of both the donors and recipients, clinical complications including delayed RBC engraft are not observed. Nevertheless, the level of isoagglutinin titer increase is reflective of

immunohematologic reconstruction after stem cell transplantation (15).

IDDI is generally not classified as a posttransplant phenomenon because of the inconsistent increases in DDI observed in transplant subjects. Nonetheless, IDDI was the subject of this study. Our overall goal was to determine why DDI increases in some posttransplant subjects. A statistically significant factor was not observed in total groups (*P* > 0.05). In patients possessing DDIs against RBCs of the recipient, however, the IDDI frequency of female patients tended to be higher compared with that of male patients (23.08 vs. 8.33%, *P* = 0.06). Similar results were observed in our previous study (11).

Although GVHD is thought to be instigated by T lymphocytes (16–18), some research groups now conjecture that B lymphocytes may play a role in GVHD (12,13). Acute GVHD is prompted by antigens expressed by antigen-presenting host cells and by inflammatory cytokines secreted by donor T lymphocytes (8). However, acute GVHD is triggered only when host B lymphocytes express minor histocompatibility antigen to T lymphocytes of the donor (19). Chronic GVHD is an isoimmune disease that arises because of B lymphocyte dysregulation. Moreover, it is closely related to systemic autoimmune diseases and may be affected by several factors (20). Chimerism and the titer of IgG autoantibody have been reported to be related to each other in a stem cell transplanted mouse model (12,13); this may also hold true for antibodies originating from a donor. Some researchers maintain that chronic GVHD in humans can be treated by removing B lymphocytes (21,22); GVHD was successfully treated by depleting B lymphocytes in a mouse model (19). Autoimmune diseases can also be improved by removing B lymphocytes, providing support for this treatment approach for GVHD (23). Therefore, the relationship between B lymphocyte humoral immunity and GVHD may warrant investigation.

The relationship between GVHD and ABO incompatibility has been the subject of previous studies. The frequency of stage I acute GVHD was shown to increase after ABO-incompatible HSCT (8), and GVHD incidence was higher with ABO incompatibility compared with compatibility (24). Moreover, Keever-Taylor et al. (25) demonstrated that the relative risk of acute GVHD in minor incompatibility is 2-fold higher than compatibility through multivariate analysis in patients with T-cell-depleted bone marrow transplants. The relationship between ABO incompatibility and GVHD was not shown in an individualized study that focused on bone marrow (26) and peripheral blood stem cell transplantation (27). In a study on human leukocyte antigen (HLA)-compatible transplantation, Rozman et al. (28)

concluded that ABO incompatibility was not related to GVHD. Hence, given the variable results of the above-mentioned studies, determining whether ABO incompatibility increases the frequency of GVHD is difficult. Therefore, we focused on elucidating relationships between GVHD and IDDI, which are observed in ABO incompatibility and presumed to emerge by an immunomechanism rather than through ABO incompatibility.

Our previous studies (10,11) showed that changes in isoagglutinin titers in patients who underwent ABO-incompatible HSCT might have clinical implications (e.g., PRCA, hemolysis), suggesting the presence of graft-versus-plasma cell effects and immunohematologic reconstitution. In this study, we focused on another clinical implication, GVHD, which was divided into acute and chronic GVHD to investigate the relationship between IDDI and GVHD; significant differences relating to the frequency of IDDI, however, were not observed in either group. IDDI analysis on posttransplant days 30, 60, and 90 showed that patients with DDIs against RBCs of the recipient and GVHD were more inclined to have IDDI prior to posttransplant day 60 compared with groups that did not have GVHD (17.3 vs. 3.9%, $P = 0.058$). The frequency of IDDI increased 2-fold in all groups, which may be reflective of results from patients with DDIs against RBCs of the recipient. A difference was not observed in patients with DDIs that were not against RBCs of the recipient. Hence, it appears that acute GVHD may be related to only those cases in which IDDI against RBCs of the donor occurred before posttransplant day 60. Early IDDI against RBCs of the donor is therefore a likely cause of GVHD. Nevertheless, IDDI is only partially observed (17.3%) in patients with acute GVHD.

IDDI is presumed to appear clinically only when isoagglutinin increases to sufficient levels that allow antigens on the endothelium. Consequently, the possibility of acute GVHD should be considered if IDDI occurs in response to donor erythrocytes early in transplantation. Acute GVHD frequency appears to increase when IDDI occurs early, for short durations, and when the donor is young. Diverse differences may arise owing to increases in DDI, which appear after a prescribed period because acute GVHD does not emerge after a certain amount of time has elapsed. The higher frequency of acute GVHD in younger patients may be owing to an increased activity of lymphocytes relative to that found in older patients. This problem should be considered. Moreover, the frequency of IDDI in patients with DDIs against RBCs of the recipient was two times higher compared with patients with DDIs that were not against RBCs of the recipient. Hence, a fundamental difference may exist between IDDI that

respond to donor erythrocytes and IDDI that do not respond to erythrocytes of donors or recipients. In particular, only the DDIs against RBCs of the recipient showed a significant result in the cases when the intensification on the host was considered as the same as GVHD. Conversely, DDI not against RBCs of the recipient lacked significance. Moreover, patients with DDIs against RBCs of the recipient and patients with DDIs not against RBCs of the recipient differed in their durations of isoagglutinin increase (days 23 and 112, respectively) and had different frequencies of IDDI. In this study, titer increases of 4-fold or greater were regarded as the standard. However, we cannot conclude that this is entirely related to the accentuation of donor lymphocytes because of limitations in the differentiation between donor and recipient isoagglutinin (i.e., in the cases of $A \rightarrow O$, $B \rightarrow O$, $O \rightarrow A$, and $O \rightarrow B$ patients) (Table 1). This was the primary limitation of our study.

In summary, the goal of this study was to examine the relationship between DDIs and GVHD. We show that IDDI occurred in many patients after transplantation. Moreover, IDDI observed on posttransplant day 60 were correlated with acute GVHD in ABO-incompatible HSCT. The findings suggest that changes in isoagglutinin titers might have clinical implications in the occurrence of immunohematological complications such as PRCA, immune-mediated hemolysis (10,11), and acute GVHD. Furthermore, isoagglutinin titers might reflect immunohematological reconstitution after transplantation and acute GVHD owing to graft-versus-plasma cell effects. This study suggests a potential role of DDIs for humoral immunity in GVHD, which warrants further study.

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