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Autoimmune Hemolytic Anemia after Allogeneic Hematopoietic Stem Cell Transplantation: Analysis of 533 Adult Patients Who Underwent Transplantation at King's College Hospital

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A B S T R A C T

Autoimmune hemolytic anemia (AIHA) is a recognized complication of hematopoietic stem cell transplantation (HSCT); it is often refractory to treatment and carries a high mortality. To improve understanding of the incidence, risk factors, and clinical outcome of post-transplantation AIHA, we analyzed 533 patients who received allogeneic HSCT, and we identified 19 cases of AIHA after HSCT (overall incidence, 3.6%). The median time to onset, from HSCT to AIHA, was 202 days. AIHA was associated with HSCT from unrelated donors (hazard ratio [HR], 5.28; 95% confidence interval [CI], 1.22 to 22.9; $P = .026$). In the majority (14 of 19; 74%) of AIHA patients, multiple agents for treatment were required, with only 9 of 19 (47%) patients achieving complete resolution of AIHA. Patients with post-transplantation AIHA had a higher overall mortality (HR, 2.48; 95% CI, 1.33 to 4.63; $P = .004$), with 36% (4 of 11 cases) of deaths attributable to AIHA.

INTRODUCTION

The development of autoimmune diseases is an increasingly recognized complication after hematopoietic stem cell transplantation (HSCT) [1]. Depending on the type of HSCT, the effector lymphocytes can either be autologous or allogeneic in origin. After autologous HSCT, autoreactive lymphocytes targeting "self" tissue can lead to a range of autoimmune diseases affecting the hematological, endocrine, and neurological systems, as well as connective tissues. After allogeneic HSCT, autoimmunity specifically refers to allogeneic lymphocytes that target donor-derived tissue, eg, autoimmune cytopenia, or well-recognized autoantigens not specific to the donor or recipient, for instance, acetylcholine receptor in myasthenia gravis. Among autoimmune conditions that develop after HSCT, autoimmune hemolytic anemia (AIHA) is the most frequently reported [1-3].

Current estimates of the incidence of AIHA after HSCT are between 2% to 6%, affecting both adult [4-9] and pediatric [2,3,10,11] patients. Higher incidences of up to 15% to 20% have been reported [12,13], albeit in specific clinical settings and with less conventional conditioning regimens. The median time of onset of AIHA has been observed to be between 5 and 12 months after HSCT [2-8,10]. Hemolysis can be mediated by cold (IgM) or warm (IgG) autoantibodies, which has been suggested to correlate with early or late onset AIHA, respectively, reflecting the process of B lymphocyte switch from IgM to IgG production during immune reconstitution after HSCT [5].

Development of AIHA after HSCT has been associated with HSCT from unrelated donors and concurrent chronic graft-versus-host disease (GVHD) [6,9], suggesting a role for mismatched antigens in the pathogenesis of AIHA after allogeneic HSCT. Lymphocyte depletion of the donor graft with ex vivo lymphodepletion [12] or in vivo lymphodepletion using antithymocyte globulin (ATG) or alemtuzumab [7,8] have also been associated with AIHA, possibly through removal of $CD4^+$ $CD25^+$ regulatory T cells, thus favoring the expansion of autoreactive lymphocytes [14]. Other reported

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Table 1
Transplantation Characteristics of HSCT Recipients and Patients Developing Post-Transplantation AIHA

Characteristic	Total	AIHA, n (%)	*P* Value
No. of HSCTs	533	19 (3.6)	
Sex			
M	323	14 (4.3)	.23
F	210	5 (2.4)	
Age at HSCT, average, yr	49	48	
Diagnosis			
AML	204	6 (2.9)	NS
MDS	112	5 (4.5)	
NHL	63	2 (3.2)	
AA	47	2 (4.3)	
ALL	25	0	
CML	24	2 (8.3)	
CLL	19	2 (10.5)	
MPD	12	0	
HL	10	0	
MM	7	0	
Others	10	0	
HSCT type			
UD	352	17 (4.8)	.03
Sibling	181	2 (1.1)	
Source of stem cells			
Peripheral blood	446	16 (3.6)	.44
Bone marrow	52	3 (5.8)	
Cord	26	0	
–	9	0	
Condition regimen			
Alemtuzumab/ATG	422	17 (4.0)	.26
Non-alemtuzumab/ATG	111	2 (1.8)	
RIC	473	17 (3.6)	.92
Myeloablative	60	2 (3.3)	
HLA mismatch			
0	417	15 (3.6)	.37
1	68	4 (5.9)	
2	21	0	
>2	19	0	
–	8	0	
ABO mismatch			
No	251	7 (2.8)	NS
Major	111	3 (2.7)	
Minor	122	7 (5.7)	
Bidirectional	41	2 (4.9)	
ND	8	0	
Sex mismatch			
No	302	16 (5.3)	.02
Mismatch	207	3 (1.4)	
–	24	0	
Recipient CMV Status			
Positive	272	9 (3.3)	.60
Negative	238	10 (4.2)	
–	23	0	
Chronic GVHD			
Yes	142	8 (5.6)	.20
No	358	11 (3.1)	
–	33	0	

M indicates male; F, female; AML, acute myeloid leukemia; NS, not significant; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; AA, aplastic anemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphoid leukemia; MPD, myeloproliferative disease; HL, Hodgkin lymphoma; MM, multiple myeloma; RIC, reduced-intensity conditioning.
A dash indicates that data are not available.

risk factors associated with onset of AIHA include receiving HSCT for treatment of nonmalignant diseases [2,3,10], being younger at transplantation [10], receiving HSCT from peripheral blood stem cells [12], and having a short interval from diagnosis of disease to HSCT treatment [2]. The clinical significance of these risk factors remains uncertain, as they have not been consistently reported among studies.

Despite the multitude of immunosuppressive therapies used for treatment, the clinical outcome of AIHA after HSCT is variable [1,2]. Currently, there is no consensus regarding the optimal therapeutic approach. Whether AIHA contributes to increased mortality is equally controversial. Although certain studies have observed mortality of up to 50% in patients with AIHA after allogeneic HSCT [6,10], others have reported much lower mortality in children [3], and no increase in mortality was attributed to secondary autoimmune diseases after autologous [8] or cord blood HSCT [2]. At present, mortality data are lacking for adult populations undergoing HSCT for malignant diseases, the largest patient population undergoing transplantation. To better understand the risk factors, prognosis, and management of post-HSCT AIHA, we carried out a retrospective analysis of 533 allogeneic HSCTs performed at King's College Hospital between 2005 and 2011.

MATERIALS AND METHODS

Patients

Between January 2005 and November 2011, 533 allogeneic HSCTs carried out at King's College Hospital (London, UK) were included in the study. In patients who received more than 1 HSCT, each transplantation was counted as a separate event if the graft was received from a different donor, and the transplantations were counted as the same event if the graft was received from the same donor. The median follow-up period after HSCT was 31 months (range, 2.9 to 100 months).

Transplantations Protocols

The transplantation protocols were approved by the local research ethics committee and informed consent was obtained from all patients. Human leukocyte antigen (HLA) typing of recipients and donors was carried out by high-resolution molecular techniques. Patients with an HLA-matched sibling donor received the graft from the sibling; otherwise, a suitable unrelated donor (UD) was identified. The selection of conditioning regimen and source of hematopoietic stem cells were based on hematological diagnosis, donor availability, and clinical state of the patient. GVHD prophylaxis for standard conditioning regimens consisted of methotrexate with cyclosporine administered for a minimum of 6 months and tapered thereafter. In reduced-intensity conditioning regimens, the only GVHD prophylaxis was cyclosporine, administered from day 1 and tapered from day +56 in the absence of GVHD. Granulocyte-colony stimulating factor (Granocyte, 263 μg/day, Chugai Pharma, London, UK; or Neupogen 300 μg/day, AMGEN, Thousand Oaks, CA) was administered subcutaneously or intravenously from day +7 until stable neutrophil engraftment. Chimerism analysis was performed routinely on days +28, +56, +100, then 6 months after HSCT using single nucleotide polymorphism analysis. Red cells and platelets were transfused to maintain hemoglobin level and platelet count above 80 g/L and 10×10^9/L, respectively. All cellular blood products in the United Kingdom (except granulocytes) are leukodepleted at source by filtration. All patients received antiviral and antifungal prophylaxis using aciclovir and a triazole agent. Prophylaxis with azithromycin was given to donors or recipients with serological evidence of toxoplasma exposure. All patients were monitored weekly for cytomegalovirus (CMV), Epstein-Barr virus, and adenovirus DNA. The conditioning regimen and transplantation characteristics of our patients are shown in Table 1.

Definitions

AIHA was defined by positive direct agglutinin test (DAT) arising after the HSCT, with biochemical markers of hemolysis (raised serum lactate dehydrogenase, reduced haptoglobin, or spherocytes on the blood film). Hemolysis was considered significant if the drop in hemoglobin was ≥20 g/L. Cases of DAT positivity due to ABO antibodies, as well as those with history of AIHA or positive DAT before HSCT, were excluded. DAT was performed in selected patients after HSCT with appropriate clinical indications. Patients who never had DAT were presumed not to have clinically significant AIHA. Patients with positive DAT but evidence of nonimmune hemolysis, eg, microangiopathic hemolytic anemia, were also excluded. Onset of AIHA was calculated from the first documented detection of AIHA by either clinical symptoms or positive DAT. *Resolution* of AIHA was defined as normalization of hemoglobin and biochemical markers of hemolysis and independence from additional treatment. *Partial response to treatment* for AIHA was defined as an improvement in markers of hemolysis with requirement of maintenance treatment. Included within the partial response group were 2 patients who suffered relapse of their primary hematological disease, resulting in withdrawal of treatment for AIHA, and 2 additional patients who died of non-AIHA–related causes while receiving treatment for AIHA. *Refractory AIHA* was defined as failure to respond after 4 or more modalities

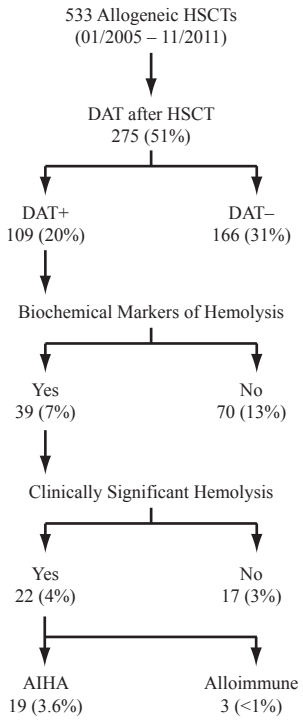

Figure 1. Identification of AIHA from 533 allogeneic HSCTs.

of treatment, including steroids, rituximab, further immunosuppressive therapies (cyclosporine, mycophenolate mofetil, azathioprine, and sirolimus) or surgical splenectomy.

Serology

All recipients and donors had ABO and Rh blood group typing (forward and reverse) performed on DiaMed gel columns. Antibody screening was performed by indirect antiglobulin test against a 3-cell panel (DiaMed, Cressier, Switzerland); antibody identification was performed against a panel of 10 cells. DAT was performed on DiaMed gel columns. The presence of IgG and C3d on red cells was demonstrated by testing with monospecific reagents at 37°C. Red cell–bound antibodies were eluted by acid elution (Gamma Elu-kit, Immucor, Norcross, GA).

Statistical Analysis

The primary endpoint was the onset of AIHA. Potential risk factors for development of AIHA were calculated initially with univariate comparisons of incidence of AIHA for each clinical strata using chi-squared test. All *P* values were 2 sided and considered significant if $<.05$. The following variables were included in univariate analysis for risk factors: recipient gender, primary hematological disease, HSCT type (unrelated versus sibling), source of stem cells (peripheral blood versus bone marrow versus cord), conditioning regimen (alemtuzumab/ATG versus non-alemtuzumab/ATG, and reduced intensity versus myeloablative), HLA mismatch between donor and recipient, ABO antigen mismatch, gender mismatch between donor and recipient, recipient CMV status, and concurrent chronic GVHD. Chronic GVHD was modeled as a time-dependent variable, whereas all other variables were modeled as categorical variables. Risk factors achieving statistical significance on univariate analysis underwent additional multivariate analysis with Cox regression to identify the most significant independent risk factors.

The secondary endpoint was mortality. The Cox regression proportional hazard model was used to compare mortality caused by AIHA against overall mortality and transplantation-related mortality. As the onset of AIHA varied over time after HSCT, AIHA was modeled as a time-dependent variable when estimating mortality. All patients alive at last follow-up who did not develop AIHA were censored. SPSS Statistics (IBM SPSS Statistics for Macintosh, Version 21.0; IBM Corp., Armonk, NY) software was used for all statistical analysis.

RESULTS

Identification of AIHA after Allogeneic HSCT

Records of 533 HSCT patients were analyzed for evidence of AIHA (Figure 1, Table 1). Of the 279 patients who had a DAT after HSCT, 109 patients tested DAT positive. Thirty-nine of 109 cases (7% of the total number of patients) had a positive DAT and positive biochemical markers of hemolysis, but only 19 of these cases exhibited clinically significant hemolysis to be classified as AIHA, resulting in an overall incidence of 3.6% (Figure 1). In the other 20 cases with positive DAT and biochemical markers of hemolysis, 3 cases exhibited alloimmune hemolysis, whereas the remaining 17 cases did not exhibit clinically significant hemolysis.

Clinical characteristics of the AIHA cases are summarized in Table 2. The median time to onset of AIHA after HSCT was 202 days (Figure 2). In 3 cases, onset of AIHA was preceded

Table 2
Clinical Characteristics of Patients with AIHA

Case No.	Diagnosis	Sex	Age at HSCT	HSCT Type			Time to AIHA, d	Chronic GVHD	Peripheral Blood Chimerism		
				Donor	Regimen	RIC/MA			UF	CD3	CD15
1	AML	F	42	UD	FBC	RIC	121	Yes	100	100	100
2	MCL	M	59	UD	BEAM C	RIC	131	Nil	95	95	100
3	AML	M	46	Sibling	FCC	RIC	135	Nil	100	86	100
4	MDS	M	28	UD	FluATG	RIC	144	Yes	98	100	100
5	AA	M	37	UD	FCC	RIC	175	Nil	99	82	100
6	CML	M	49	UD	FBC	RIC	180	Yes	-	-	-
7	AML	F	65	UD	FBC	RIC	183	Yes	99	77	99
8	AA	M	21	UD	FCC TBI	RIC	187	Yes	100	100	-
9	CLL	M	60	UD	FMC	RIC	193	Yes	100	98	100
10	AML	F	43	UD	FBC	RIC	202	Yes	95	96	100
11	MDS	F	54	UD	FBC	RIC	204	Nil	81	27	83
12	CML	M	34	UD	BU CY TBI	MA	209	Nil	100	-	-
13	AML	M	33	UD	BU CY TBI	MA	220	Nil	100	-	-
14	MDS	M	58	UD	FBC	RIC	220	Yes	100	-	-
15	AML	M	62	UD	FBC	RIC	253	Nil	72	5	82
16	CLL	M	51	UD	FMC	RIC	315	Nil	100	100	100
17	MDS	F	67	UD	FBC	RIC	340	Nil	99	82	100
18	MDS	F	44	UD	FBC	RIC	378	Nil	100	-	-
19	NHL	M	46	Sibling	BEAM C	RIC	569	Nil	100	82	100

MA indicates myeloablative; UF, unfractionated; FBC, fludarabine/busulfan/alemtuzumab; BEAM C, carmustine/cytarabine/etoposide/melphalan/alemtuzumab; FCC, fludarabine/cyclophosphamide/alemtuzumab; Flu, fludarabine; TBI, total body irradiation; FMC, fludarabine/melphalan/alemtuzumab; BU, busulfan; CY, cyclophosphamide.
A dash indicates that data are not available.

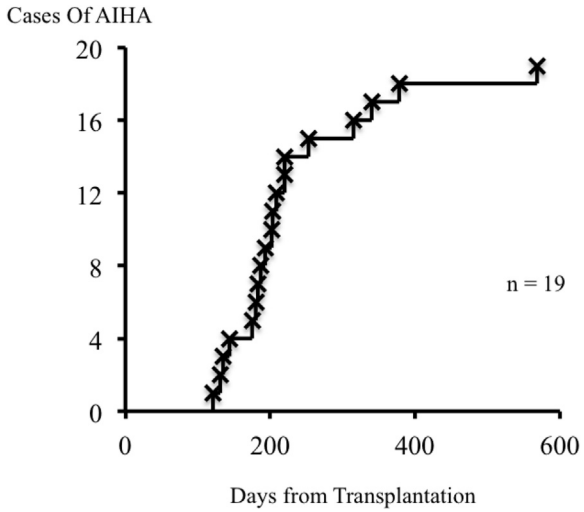

Figure 2. Event plot showing days since HSCT until the onset of clinically detectable AIHA.

within 1 month by an infective episode (adenovirus, CMV, and pneumonia of undetermined etiology). At the onset of AIHA, one half of the patients were receiving immunosuppressive therapies, either as GVHD treatment or prophylaxis. Unfractionated peripheral blood, CD3, and CD15 chimerism were tested within 1 month of onset of AIHA in 18 of 19 patients; of those, 16 had 95% to 100% unfractionated peripheral blood donor chimerism, with 77% to 100% CD3/CD15 chimerism (Table 2). Case 11 had 81% unfractionated peripheral blood donor chimerism at onset of AIHA and had concurrent relapse of MDS. Case 15 had 72% unfractionated peripheral blood chimerism at onset of AIHA. In both cases, where mixed donor/recipient chimerism was present, we cannot exclude the possibility that a recipient-versus-donor alloimmune hemolysis could have contributed to the immune-mediated hemolysis.

Risk Factors for AIHA

Transplantation characteristics were compared between allogeneic HSCT recipients without AIHA and those who developed AIHA after HSCT (Table 1). Univariate analysis showed that AIHA was significantly associated with HSCT from an unrelated donor (UD 4.8% versus sibling donor 1.1%, $P = .03$), and concordant gender between the donor and the recipient (no gender mismatch 5.3% versus gender mismatch 1.4%, $P = .02$). Multivariate analysis using Cox regression model showed that HSCT from UD remained statistically significant ($P = .026$; hazard ratio [HR], 5.28; 95% confidence interval [CI], 1.22 to 22.9), whereas concordant gender was of borderline significance ($P = .045$; HR, 3.52; 95% CI, 1.03 to 12.1). No significant association was observed between AIHA and the following variables: recipient gender, primary hematological disease, source of hematopoietic stem cells, conditioning regimen (alemtuzumab/ATG versus non-alemtuzumab/ATG, and reduced intensity versus myeloablative), HLA mismatch between donor and recipient, ABO antigen mismatch, recipient CMV status, and concurrent chronic GVHD.

Serological Analysis of AIHA Patients

In 18 of 19 cases, IgG antibodies were present on red cells, with 15 of 18 also being positive for C3d (Figure 3A). Serological details of the positive DAT for case 9 were not available. Elution studies were performed in 7 patients: panreactive antibodies were detected in 6 of 7 eluates, but only 1 of 7 eluates contained an anti-Rh antibody (anti-e), likely to be an *auto*antibody as the patient (number 13) also

A

Case	DAT(IgG/C3d)	Antibody Identification		Blood Group	
		Serum	Eluate	Donor	Recipient
1	IgG 4+ C3d 1+	ND	Pan	A+	B+
2	IgG 3+ C3d 3+	Pan, Anti-K	Pan	O+ K-	A+ K-
3	IgG 3+ C3d 3+	ND	-	A+	A+
4	IgG 1+ C3d 4+	Pan, Anti-E	-	A+	A+
5	IgG 3+ C3d 4+	Pan, Anti-C, -D	Pan	A-	B+
6	IgG 3+ C3d 3+	ND	Pan	O-	A+
7	IgG 4+ C3d 1+	ND	-	O+	B+
8	IgG 3+ C3d 3+	ND	-	O-	O+
9	Positive	Anti-E, -K	-	B+	O+
10	IgG 4+ C3d 3+	Anti-E	-	A+	A-
11	IgG 3+ C3d 3+	Anti-C, -E	-	A+	AB+
12	IgG 4+ C3d 2+	Pan, Anti-E	Pan	O+	A+
13	IgG 4+ C3d 3+	Anti-E	Anti-e	A+	A+
14	IgG 3+ C3d 4+	Anti-E, -Cw	-	B+	O+
15	IgG 4+	ND	-	A+	A+
16	IgG 3+ C3d 3+	ND	-	O+	O+
17	IgG 3+	ND	-	O-	A+
18	IgG 4+ C3d 2+	Pan, Anti-Cw	Pan	B+	O+
19	IgG 1+	Anti-D, -E	-	O-	A+

B

	Total Cases	Positive Serum Antibody (%)
AIHA	19	11 (58)
DAT -	157	6 (4)

$P < .0001$

C

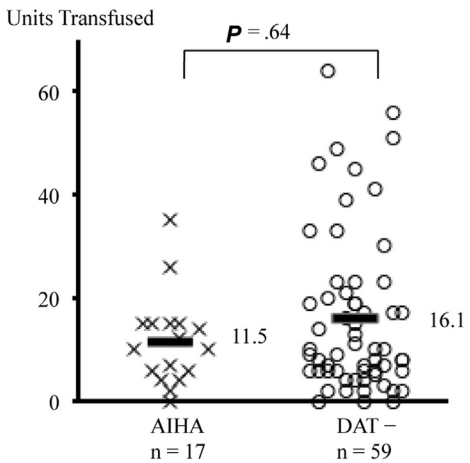

Figure 3. Serology investigations of AIHA patients. (A) Results of DAT, antibody specificities from the serum, and red cell elution studies and blood group of the donor and recipient. (B) Number of cases with antibodies identified in the serum in AIHA population compared with HSCT population that tested negative in the direct antiglobulin test. (C) Red cell units transfused in AIHA patients between the HSCT to the onset of AIHA, compared with DAT-negative patients who received transfusions over a similar time period. Average red cell units transfused as indicated. Pan indicates panreactive; ND, not detected; –, data not available.

Table 3
Clinical Course for Patients with AIHA

Case No.	Treatment	Clinical Outcome
1	Prednisolone, rituximab, cyclosporine	Resolved.
2	Prednisolone	Resolved.
3	Prednisolone, IVIg, rituximab, azathioprine, danazol	AIHA controlled with maintenance prednisolone and danazol.
4	Prednisolone, rituximab	Resolved. Died (relapsed MDS).
5	Prednisolone	Resolved
6	Prednisolone, rituximab, cyclosporine, splenectomy	Refractory AIHA. Died (AIHA).
7	Prednisolone, IVIg, rituximab	Resolved.
8	Prednisolone, rituximab, plasma exchange, splenectomy	Refractory AIHA. Died (AIHA and sepsis).
9	Prednisolone	AIHA initially resolved with treatment. Died (relapsed CLL with AIHA).
10	Prednisolone, IVIg, cyclosporine	AIHA responded to treatment but stopped due to relapsed AML. Died (AML relapse).
11	Prednisolone	AIHA responded to prednisolone but stopped due to MDS relapse. Died (MDS relapse).
12	IVIg	Resolved.
13	Prednisolone, IVIg, rituximab, plasma exchange, splenectomy, sirolimus	Refractory AIHA. Died (AIHA).
14	Prednisolone, IVIg, rituximab, cyclosporine	AIHA responded to treatment. Died (while on reducing doses of prednisolone).
15	Prednisolone, rituximab	Resolved. Died (sepsis and encephalopathy).
16	Prednisolone, IVIg, rituximab, cyclosporine, mycophenolate	Resolved.
17	Prednisolone, rituximab	AIHA responded to treatment. Died (while on reducing doses of prednisolone).
18	Prednisolone, IVIg, rituximab, mycophenolate	Refractory AIHA. Died (sepsis, AIHA).
19	Prednisolone, rituximab	Resolved.

The causes of death are shown in brackets under clinical outcome.

developed an *allo* anti-E. In addition to red cell membrane–bound antibodies, 11 patients had antibodies in the serum: panreactive (auto) antibodies were present in 5, whereas antibodies against Rh and Kell antigens were found in the serum of all 11 patients (Figure 3A).

We postulate that Rh antibodies in the serum of 11 patients were donor-derived *allo*antibodies that remained unbound to antigens on donor-derived red cells. In support of this hypothesis, patients numbered 5 and 19, initially RhD positive, received their graft from RhD-negative donors (Figure 3A); they developed an anti-D after achieving engraftment (unfractionated chimerism, 100%; CD3, 82%; and CD15, 100%). Thus, these examples of anti-D were alloantibodies of donor origin, with immunization probably occurring after exposure to RhD antigen on residual host erythrocytes. Furthermore, anti-K detected in patient number 2 was also an alloantibody, as both the donor and recipient had *kk* genotype; donor origin of the antibody is implied because of a 100% donor chimerism at the time of testing, whereas immunization probably occurred after transfusion of a K-positive red cell unit.

We assessed whether the high frequency of alloantibodies observed among the patients with AIHA was a common feature after HSCT by analyzing the patients who tested negative for DAT (Figure 3B). We found that only 4% of patients with a negative DAT developed alloantibodies, in contrast to 58% of patients with AIHA. To rule out the possible bias due to higher transfusion rate of AIHA patients, we compared the number of transfused red cell units over a similar time period before the onset of AIHA between AIHA cases and DAT-negative cases, and we observed no significant difference between these 2 groups (Figure 3C).

Treatment and Outcome

The majority of AIHA patients were treated with more than 1 therapeutic agent; 5 of 19 patients (26%) received single-agent therapy, 4 (21%) received 2 agents, and the remaining 10 patients (53%) received 3 or more agents. The specific course of treatment for each patient is shown in Table 3. Prednisolone (starting dose 1 mg/kg) was used as first-line therapy in 18 cases, with rituximab in 13 cases as second-line or third-line treatment (after a trial of intravenous immunoglobulin [IVIg]). Treatments in addition to rituximab were required in 8 cases; including combinations of plasma exchange (2 cases); immunosuppressive agents including cyclosporine, mycophenolate mofetil, azathioprine, and sirolimus (7 cases); and splenectomy (3 cases).

Overall, the response to treatment was variable. Complete resolution of AIHA was achieved in 9 of 19 cases (47%). Of these 9 cases, 1 resolved with IVIg alone, 2 with prednisolone alone; 4 required the addition of rituximab and 2 required rituximab with further immunosuppression (cyclosporine and mycophenolate mofetil). Partial response was seen in 6 of 19 patients (32%), of whom 2 died of relapsed primary hematological disease during treatment, necessitating discontinuation of immunosuppression. Two patients died of non-AIHA–related causes during AIHA treatment; 1 developed relapsed AIHA after initial response and 1 required ongoing maintenance steroids. Refractory AIHA was observed in 4 of 19 cases (21%), including the 3 who underwent surgical splenectomy that failed to control the hemolysis.

At the time of analysis, 9 of the 19 AIHA patients were alive. The median survival from onset of AIHA was 487 days (range, 26 to 1977 days). AIHA was a major cause to death in 4 patients: 2 died directly as a result of AIHA and in 2 other cases, sepsis also contributed to death. Four patients died because of disease relapse and 2 died while on prednisolone treatment. To account for the late onset of AIHA after HSCT, we used the Cox regression model to evaluate AIHA as a time-dependent risk factor, whereby association between mortality and AIHA is analyzed from the time of AIHA onset rather than spanning the total follow-up period after HSCT. Within this model, we tested additional variables that we found earlier to be associated with AIHA (HSCT from unrelated donors, donor/recipient gender concordance) in a multivariate analysis to exclude confounding factors influencing mortality (Table 4). The results indeed showed AIHA to be the only risk factor associated with both increased overall mortality (HR, 2.48; 95% CI, 1.33 to 4.63; $P = .004$) and increased transplantation-related mortality (HR, 4.38; 95% CI, 1.96 to 9.77; $P < .001$). Therefore, we conclude that patients who developed AIHA as a complication of HSCT has

Table 4
Cox Regression Multivariate Analysis of Potential Risk Factors for Overall and Transplantation-Related Mortality

	Overall Mortality			Transplantation-Related Mortality		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value
AIHA	2.48	1.33-4.63	.004	4.38	1.96-9.77	<.001
Unrelated donor	.94	.72-1.22	.63	.80	.55-1.18	.27
Gender concordance	.88	.68-1.14	.32	.68	.48-.97	.03

AIHA was modeled as time-dependent variable; unrelated donor and gender concordance (between donor/recipient) were modeled as time-independent variables.

significantly increased risk of mortality compared with patients who did not develop AIHA.

DISCUSSION

In our single-center retrospective analysis of 533 adult patients who underwent allogeneic HSCT, we report the overall incidence of AIHA after HSCT is 3.6%. This is in agreement with the previously reported incidence for comparable populations [4-6,9]. One study reported the incidence of AIHA after HSCT to be as high as 15% in adult patients with chronic myeloid leukemia treated with HSCT [13]. However, the majority of the hemolysis cases coincided with relapsed chronic myeloid leukemia and resolved with donor lymphocyte infusion, suggesting the immune-mediated hemolysis was not true AIHA but more likely recipient-versus-donor alloimmune hemolysis. None of the 26 cord blood HSCTs developed AIHA, which likely reflects the low number of cord HSCT performed at our center, where only 12 of 26 patients survived beyond 202 days (median onset for AIHA), combined with a reported incidence of AIHA after cord HSCT to be only 5% to 6.6% [2,9].

Nearly two thirds of HSCT patients with a positive DAT exhibited no biochemical or clinical evidence of hemolysis. Nevertheless, 17 patients (3% of total HSCT analyzed) had positive DAT with biochemical markers of hemolysis but without significant anemia (Figure 1). In 3 of 17 cases, there was evidence of microangiopathic hemolytic anemia, which could account for the positive markers of hemolysis with a probable incidental positive DAT. However, we cannot exclude the possibility that these patients had mild or compensated AIHA, and that AIHA after HSCT could be more common than we estimated, but in some cases the hemolysis is transient or self-resolving with little clinical impact or obscured by concurrent conditions.

Patients with the highest risk of developing AIHA were those who received HSCT from unrelated donors, consistent with results from other studies [2,3,6,10]. We hypothesized that this reflected the donor immune system reacting to mismatched antigens of the recipient, similar to the pathogenesis of GVHD. However, we did not observe an increased incidence of AIHA in patients receiving HSCT from donors mismatched at class I/II HLA antigens or ABO antigens. In addition, we observed no association between AIHA and chronic GVHD, a complication known to be associated with increasing degree of mismatch in HLA antigen between the donor and recipient. Other studies in adult HSCT have identified chronic GVHD as a risk factor for AIHA [6,9]; the disparity with our study may simply reflect the overall low number of AIHA cases in the studies, but it raises the possibility that the mechanism underlying AIHA is distinct from that causing GVHD. In our patient cohort, as AIHA occurred at the time when more than 95% blood cells were of donor origin, we speculate that aberrant immune reconstitution of the donor HSCT within the foreign environment of the recipient may favor the expansion of autoreactive B cells. A possible mechanism could involve a delay in B cell recovery after HSCT leading to high levels of circulating plasma B cell activating factor, which has been associated with aberrant B cell homeostasis [15-18].

Studies of autologous HSCT for treatment of autoimmune diseases have identified intense lymphodepletion from using conditioning regimens containing alemtuzumab/ATG [7,8] as a risk factor for development of post-HSCT autoimmune diseases. Our study along with others failed to support this finding [2,3,6,10,12]. This discrepancy could reflect the different indications for HSCT (malignant diseases versus autoimmune diseases) and donor source of stem cells (allogeneic or autologous). However, as the majority of patients in our study received alemtuzumab/ATG as part of the conditioning (79%) with reduced-intensity conditioning regimens (89%), this could have been a limiting factor in identifying these variables as statistically significant risk factors. We also found concordant gender between the donor and recipient of the HSCT to be weakly associated with the onset of AIHA, although this will need further validation in future studies.

Warm-reacting panreactive autoantibodies were the most frequently identified red cell antibodies in our study, in agreement with other reports [5,6,10,12,13]. Chimerism studies in our patients suggest that post-HSCT AIHA is a "donor-against-donor" phenomenon; however, the exact role of the host immune system, eg, antigen-presenting cells, is poorly understood. Surprisingly, 58% AIHA patients also had antibodies against Rh/Kell antigens which, as detailed in the results section, appear to be alloantibodies. Chen et al. [5] made a similar observation that anti-Rh antibodies were found predominantly in the serum but not the eluates. An alternative explanation could be that at least some of the apparent alloantibodies were, in fact, red cell *auto*antibodies that mimicked alloantibodies by their reactions. Issitt et al. [19] found that nearly one half of apparent alloantibodies, detected in patients with positive DAT and serum panagglutinins were autoantibodies that mimicked alloantibodies with specificities within the Rh system. Association between allo- and autoimmunization has been described in patients requiring multiple blood transfusions [20-23]; however, there was no significant difference between the number of red cell units transfused over a similar time period before AIHA onset between the AIHA and non-AIHA population in our study. The high frequency of alloimmunization in our patients may be a result of the same defect in immune tolerance that underlies AIHA. Murine models have implicated $CD4^+$ $CD25^+$ regulatory T cells, whose depletion resulted in induction of both AIHA [24] and alloimmunization [25].

Current evidence [2,6,10,26-29] suggests the AIHA that develops after HSCT is more refractory to treatment compared with primary AIHA, where the majority of patients respond to steroids as first-line therapy [30]. We report a similar outcome in our study, where only 10% (2 of 19) of AIHA patients were treated successfully with prednisolone alone and 74% (14 of 19) required multiple therapeutic agents. Although AIHA in patient number 12 resolved with IVIg monotherapy, it is possible that the AIHA in this patient was transient in nature and would have resolved without additional treatment. The most effective treatment consisted of rituximab in combination with prednisolone or other immunosuppressive agents, which achieved resolution of AIHA in 46% (6 of 13) of patients treated with this regimen.

Other studies have noted similar efficacy of rituximab in treatment for post-HSCT AIHA [2,3,29]. Furthermore, a recent randomized controlled trial suggested benefit in using prednisolone and rituximab together over prednisolone alone as first-line therapy in the treatment of primary AIHA [31]. Taken together with our observations, there may be a rationale for combination treatment containing rituximab as first-line therapy for post-HSCT AIHA.

The overall mortality rate for our AIHA patients was high at 53% (10 of 19 cases), with AIHA as a cause of death in 36% of deceased patients. Although higher than expected mortality has been previously observed in transplantation patients who develop AIHA, evidence regarding whether AIHA contributes to increased mortality in these patients is lacking. We have shown that AIHA after HSCT indeed results in an increased risk of mortality with over 2-fold higher overall mortality in the patients with AIHA compared with those without. Our finding is in contrast with 2 previous studies by Daikeler et al. [2,8] that reported no mortality difference attributable to secondary autoimmune complications after autologous or cord HSCT. However, we note that in 1 of these studies [2] the major cause of death (5 of 12 cases) was indeed secondary AIHA or Evans syndrome (combination of AIHA and idiopathic thrombocytopenic purpura). Thus, although Daikeler et al. analyzed mortality for all autoimmune complications, we focused solely on AIHA, which could have a worse clinical outcome compared with other autoimmune diseases that develop after HSCT.

In conclusion, AIHA is a late complication of allogeneic HSCT that develops, on average, 202 days after transplantation. Patients are at higher risk of developing AIHA if they received HSCT from unrelated donors and potentially if the donor and recipient are of the same gender. Post-HSCT AIHA is frequently resistant to treatment and confers decreased overall survival. Given the poor overall response with single-agent therapy, prospective studies that explore the benefit of combination first-line therapy with steroids and rituximab are warranted.

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REFERENCES

1. Holbro A, Abinun M, Daikeler T. Management of autoimmune diseases after haematopoietic stem cell transplantation. *Br J Haematol*. 2012; 157:281-290.
2. Daikeler T, Labopin M, Ruggeri A, et al. New autoimmune diseases after cord blood transplantation: a retrospective study of EUROCORD and the Autoimmune Disease Working Party of the European Group for Blood and Marrow Transplantation. *Blood*. 2013;121:1059-1064.
3. Faraci M, Zecca M, Pillon M, et al. Autoimmune hematological diseases after allogeneic hematopoietic stem cell transplantation in children: an Italian multicenter experience. *Biol Blood Marrow Transplant*. 2014;20: 272-278.
4. Drobyski WR, Potluri J, Sauer D, Gottschall JL. Autoimmune hemolytic anemia following T cell-depleted allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 1996;17:1093-1099.
5. Chen FE, Owen I, Savage D, et al. Late onset haemolysis and red cell autoimmunisation after allogeneic bone marrow transplant. *Bone Marrow Transplant*. 1997;19:491-495.
6. Sanz J, Arriaga F, Montesinos P, et al. Autoimmune hemolytic anemia following allogeneic hematopoietic stem cell transplantation in adult patients. *Bone Marrow Transplant*. 2007;39:555-561.
7. Loh Y, Oyama Y, Statkute L, et al. Development of a secondary autoimmune disorder after hematopoietic stem cell transplantation for autoimmune diseases: role of conditioning regimen used. *Blood*. 2007; 109:2643-2648.
8. Daikeler T, Labopin M, Di Gioia M, et al. Secondary autoimmune diseases occurring after HSCT for an autoimmune disease: a retrospective study of the EBMT Autoimmune Disease Working Party. *Blood*. 2011; 118:1693-1698.
9. Sanz J, Arango M, Carpio N, et al. Autoimmune cytopenias after umbilical cord blood transplantation in adults with hematological malignancies: a single-center experience. *Bone Marrow Transplant*. 2014;49: 1084-1088.
10. O'Brien TA, Eastlund T, Peters C, et al. Autoimmune haemolytic anaemia complicating haematopoietic cell transplantation in paediatric patients: high incidence and significant mortality in unrelated donor transplants for non-malignant diseases. *Br J Haematol*. 2004;127:67-75.
11. Page KM, Mendizabal AM, Prasad VK, et al. Posttransplant autoimmune hemolytic anemia and other autoimmune cytopenias are increased in very young infants undergoing unrelated donor umbilical cord blood transplantation. *Biol Blood Marrow Transplant*. 2008;14:1108-1117.
12. Horn B, Viele M, Mentzer W, et al. Autoimmune hemolytic anemia in patients with SCID after T cell-depleted BM and PBSC transplantation. *Bone Marrow Transplant*. 1999;24:1009-1013.
13. Cwynarski K, Goulding R, Pocock C, et al. Immune haemolytic anaemia following T cell-depleted allogeneic bone marrow transplantation for chronic myeloid leukaemia: association with leukaemic relapse and treatment with donor lymphocyte infusions. *Bone Marrow Transplant*. 2001;28:581-586.
14. Daikeler T, Tyndall A. Autoimmunity following haematopoietic stem-cell transplantation. *Best Pract Res Clin Haematol*. 2007;20:349-360.
15. Jacobson CA, Sun L, Kim HT, et al. Post-transplantation B cell activating factor and B cell recovery before onset of chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2014;20:668-675.
16. Sarantopoulos S, Stevenson KE, Kim HT, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood*. 2009;113:3865-3874.
17. Lesley R, Xu Y, Kalled SL, et al. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity*. 2004;20:441-453.
18. Thien M, Phan TG, Gardam S, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity*. 2004;20:785-798.
19. Issitt PD, Combs MR, Bumgarner DJ, et al. Studies of antibodies in the sera of patients who have made red cell autoantibodies. *Transfusion*. 1996;36:481-486.
20. Young PP, Uzieblo A, Trulock E, et al. Autoantibody formation after alloimmunization: are blood transfusions a risk factor for autoimmune hemolytic anemia? *Transfusion*. 2004;44:67-72.
21. Ahrens N, Pruss A, Kähne A, et al. Coexistence of autoantibodies and alloantibodies to red blood cells due to blood transfusion. *Transfusion*. 2007;47:813-816.
22. Sigler E, Shvidel L, Yahalom V, et al. Clinical significance of serologic markers related to red blood cell autoantibodies production after red blood cell transfusion-severe autoimmune hemolytic anemia occurring after transfusion and alloimmunization: successful treatment with rituximab. *Transfusion*. 2009;49:1370-1374.
23. Guirat-Dhouib N, Mezri M, Hmida H, et al. High frequency of autoimmunization among transfusion-dependent Tunisian thalassaemia patients. *Transfus Apher Sci*. 2011;45:199-202.
24. Mqadmi A, Zheng X, Yazdanbakhsh K. CD4+CD25+ regulatory T cells control induction of autoimmune hemolytic anemia. *Blood*. 2005;105: 3746-3748.
25. Yu J, Heck S, Yazdanbakhsh K. Prevention of red cell alloimmunization by CD25 regulatory T cells in mouse models. *Am J Hematol*. 2007;82: 691-696.
26. Bashey A, Owen I, Lucas GF, et al. Late onset immune pancytopenia following bone marrow transplantation. *Br J Haematol*. 1991;78:268-274.
27. Ship A, May W, Lucas K. Anti-CD20 monoclonal antibody therapy for autoimmune hemolytic anemia following T cell-depleted, haplo-identical stem cell transplantation. *Bone Marrow Transplant*. 2002;29: 365-366.
28. Corti P, Bonanomi S, Vallinoto C, et al. Rituximab for immune hemolytic anemia following T- and B-Cell-depleted hematopoietic stem cell transplantation. *Acta Haematol*. 2003;109:43-45.
29. Raj K, Narayanan S, Augustson B, et al. Rituximab is effective in the management of refractory autoimmune cytopenias occurring after allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2005;35: 299-301.
30. Barros MMO, Blajchman MA, Bordin JO. Warm autoimmune hemolytic anemia: recent progress in understanding the immunobiology and the treatment. *Transfus Med Rev*. 2010;24:195-210.
31. Birgens H, Frederiksen H, Hasselbalch HC, et al. A phase III randomized trial comparing glucocorticoid monotherapy versus glucocorticoid and rituximab in patients with autoimmune haemolytic anaemia. *Br J Haematol*. 2013;163:393-399.