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Abstract

Background : It has been controversial whether HLA antibodies cause hemolytic transfusion reactions (HTR) or shortened RBC survival. We report a patient who had two episodes of HTR, the latter of which was likely due to RBC-reactive HLA antibodies.

Case Report : A 77-year-old woman, admitted for gastric varix rupture, had no RBC irregular antibodies detected before transfusion. On hospital day 12, after transfusion of two units of RBCs and two units of FFP, the first delayed hemolytic episode occurred and anti-E, anti-c, anti-Jk^a and unidentified RBC-reactive antibodies were detected in serum from day 14. Two further units of compatible RBCs were transfused using a leukocyte-reduction filter on days 19 and 22. After 4 hours of starting a transfusion on day 22, the patient had fever, and a second hemolytic episode was recorded. Multireactive HLA antibodies (reactive against 20 of 20 donor panel lymphocytes) were detected in sera from day 15 to day 21. These HLA antibodies reacted strongly with HLA-A2 and HLA-B7 antigens, corresponding to Bg^c and Bg^a antigens on RBCs, respectively. RBCs transfused on day 22 were found to be HLA-A2 by genotyping.

Conclusion : Strong HLA alloantibodies in this recipient appear to have caused a HTR. It is suggested that HLA antibodies be considered in patients with unexplained HTRs.

Introduction

HLA antigens are expressed on immature red cells and reticulocytes[1], but most of them disappear as RBCs mature[1]. However, several HLA antigens persist on mature RBCs, and they are known as Bg (Bennett-Goodspeed) antigens[2]. Bg^a, Bg^b and Bg^c antigens correspond to HLA-B7, HLA-B17 and HLA-A28/A2, respectively[2,3]. Generally, the antibodies to these antigens (RBC-reactive HLA antibodies; anti-Bg) are not considered clinically significant with regard to RBC transfusion therapy. However, there are reports of HLA antibodies shortening RBC survival[4-6]. Benson[7] recently described a patient who experienced both a delayed hemolytic transfusion reaction (DHTR) and then an acute, intravascular HTR due to HLA antibodies including anti-HLA-A2, -A28, -B7, and B7 cross-reactive group (CREG).

We report a patient who had a DHTR associated with multiple RBC antibodies and an acute HTR associated with RBC-reactive HLA antibodies.

Case Report

A patient, a 77-year-old Japanese woman with hepatic cirrhosis, had a medical history of a surgery for ovarian cyst at 40 years of age and one pregnancy. No prior transfusion history was known. She underwent endoscopic sclerotherapy for gastric varix rupture. At admission, the RBC antibody screen was negative. Two units of irradiated-RBCs (both stored for 5 days) and two units of FFP were transfused (days 0 and 1) without clinical complications.

Hemoglobinuria (3+), and hematuria showing the presence of many intact RBCs (>100/field) in urine because of catheter inserted, was observed between days 12 and 13. There were increases in indirect bilirubin (I-Bil: 4.7mg/dL) and lactate dehydrogenase (LDH: 879U/L) levels, and decrease in hemoglobin level (Hb: 6.8g/dL) (see Fig.1). Anti-E, anti-c, anti-Jk^a and unidentified RBC-reactive antibodies were detected in the sample of day 14. DAT was negative with her samples of days 9, 13 and 14.

On days 19 and 22, two units of crossmatch-compatible c-, E-, Jk(a-) irradiated-RBCs (stored for 7 and 8 days, respectively) were transfused using a leukocyte-reduction filter. RBCs were transfused without any clinical evidence of hemolysis on day 19. For RBCs transfusion on day 22, crossmatch test was done just prior to the transfusion. However, 4 hours after the start of transfusion on day 22, her temperature rose from 37C to 38.5C. Levels of I-Bil (4.3mg/dL) and LDH (680U/L) were moderately increased 6 hours after the start of transfusion. Haptoglobin level was not evaluated. Between days 23 and 24, hemoglobinuria (3+), evidenced by the scarce

presence (1-4/field) of RBCs in urinalysis, was observed. HLA antibodies (reactive with all lymphocytes of 20 donors) were detected in her sera from days 15 to 21. DAT was weakly positive for the sample of day 26. Antibody screen was repeated on days 22 and 26, and no further irregular antibody was found. The second HTR was possibly caused by the patient's HLA-antibodies; no other cause for the hemolytic reaction was identified; her renal function was not affected. She left the hospital on day 49.

Materials and methods

Red cell antibody test

An RBC antibody screen used saline, bromelin, and indirect antiglobulin tests (IAT) using polyethylene glycol (PEG-IAT) and saline-IAT (60min incubation at 37C). Cell panels for antibody screening and identification were used with commercially prepared kits (Surgescreen, Diego A cells, Resolve Panel A and B, Ortho Clinical Diagnostics, NJ, USA, and Panocell-16, Immucor, Inc, GA, USA). Crossmatch tests were performed using standard tube methods for agglutination test, albumin-IAT and saline-IAT. Direct antiglobulin test (DAT) used anti-human globulin reagents including polyspecific, anti-IgG, and anti-C3b/C3d (all, Immucor Inc).

Biweekly antibody screens are required officially to be performed in Japan when no adverse transfusion reactions are seen.

Antibody was eluted from the patient's RBCs using dichloromethane dichloropropan (DT- Ⅱ reagent, Ortho Clinical Diagnostics). To denature HLA class I antigens present on RBCs, chloroquine treatment[8] was used.

The patient's serum was treated with 0.01 mol/L dithiothreitol (DTT) (Wako Pure Chemical Industries, Osaka, Japan) at 37C for 15 min to distinguish IgG class antibody from IgM antibody.

HLA antibody test and HLA typing

HLA antibodies were screened against a panel of 20 donors, covering >95% of the Japanese HLA phenotypes, by the lymphocyte cytotoxicity test (LCT)

and anti-human globulin-enhanced LCT (AHG-LCT). Specificity of antibodies was confirmed against complementary donors. Antibody titer was determined by the maximally diluted sera which showed a toxicity of >20% against target cells. HLA typing of the patient, her son and the 4 RBC donors was performed by a polymerase chain reaction with sequence specific oligonucleotide probes (Dynal RELI SSO HLA-A, -B Typing Kit, Dynal Biotech, Wirral, UK).

Monocyte monolayer assay (MMA)

A modification of the method described by Arndt and Garratty[9] was used. Mononuclear cells from normal volunteer donors were separated by centrifugation over a Ficoll-sodium diatrizoate density gradient (Lymphocepal-I, Immuno-Biological Laboratories, Gunma, Japan). After washing, the mononuclear cells were suspended in culture media (RPMI Medium 1640, GIBCO/Invitrogen Life Technologies, NY, USA), containing 5% fetal calf serum (5%FCS-RPMI), and added to glass slides (Micro Slide Glass, Matsunami Glass Ind., Osaka, Japan). After one hour incubation at 37C in a CO_2 incubator containing 5% CO_2, the supernatant containing nonadherent lymphocytes was removed via pipette, and sensitized RBCs [patient's serum plus E-, c-, Jk(a-), Bg(a+) red cells or E-, c-, Jk(a-), Bg(a-) RBCs plus fresh normal serum as a source of complement] were incubated 60 min at 37C then washed with saline and suspended in 5%FCS-RPMI. After two-hour incubation, which is one-hour longer method than Arndt and Garratty's[9], at 37C, non-adherent or non-phagocytosed RBCs were removed

with 37C pre-warmed 5%FCS-RPMI via pipette. Cut-off value for MMA is 1% in our laboratory.

The slides were stained with a May-Giemsa stain and observed microscopically. Six hundred monocytes were counted, and the percentage of monocytes with RBCs adhering and/or phagocytosed was determined.

Results

At admission, the RBC antibody screen was negative. However, on day 14, anti-E (titer of 8 by saline test and 64 by PEG-IAT against DccEE Jk(a-b+) cells), anti-c (negative by saline test and a titer of 1 by PEG-IAT against dccee Jk(a-b+) cells), anti-Jk^a (negative by saline test and a titer of 4 by PEG-IAT against DCCee Jk(a+b-) cells) and unidentified RBC-reactive antibodies (titers of 1 and 4 by PEG-IAT) were detected. Two units of RBCs transfused on days 0 and 1 were later found to be incompatible with anti-E, anti-c or anti-Jk^a in the patient's serum (Table1). Blood samples obtained on days 9, 13 and 14 were DAT-negative. Anti-E (titer of 64 by PEG-IAT) of serum of day 14 was not influenced with the treatment of DTT. No antibodies were demonstrated in the eluate prepared from the patient's RBCs. It appeared that transfused RBCs were destroyed in this interval, as hemoglobinuria was observed at day 12.

For days 19 and 22 transfusions, donors were selected who were compatible with the above RBC antibodies and matched with the patient's main RBC antigens (Rh, Kidd, Duffy, MNSs, and Diego). The DAT on the day 26 sample showed was weakly positive by anti-IgG or polyspecific serum but negative by anti-C3. An eluate from the patient's RBCs was weakly reactive with 8 of 11 cells of a panel (Resolve Panel A, Ortho Clinical Diagnostics) and the RBCs transfused on day 22 (Donor 4). However, specificity of the antibody(ies) in the eluate could not be determined. No FFP transfused was tested whether they contained alloantibodies. No HLA antibodies were found before the first transfusion on day 0 and day 1 in the

serum of the patient. Both sera of days 15 and 21 were LCT and AHG-LCT positive strongly and contained broadly reactive antibodies (reactive with 20 of 20 donor panel cells; titers of 128-512 by AHG-LCT against HLA-B7 corresponding to Bg^a, and 5120 against HLA-A2 corresponding to Bg^c) were found. Both RBCs donors of days 0 (Donor 1) and 1 (Donor 2) had HLA-A2 and HLA-B61 (CREG for HLA-B7). And, days 19 and 22 RBC donors (Donor 3 and Donor 4) had HLA-B48 (CREG for HLA-B7) and HLA-A2, respectively (Table 1).

The patient's sera of days 15 and 21 were reactive with all four RBCs with [E-, c-, Jk(a-) and Bg(a+)] (Immucor Inc) and 3 of 7 RBCs with [E-, c-, Jk(a-) and Bg unknown] (Ortho Clinical Diagnostics). This reactivity of the serum was eliminated after absorption with human pooled platelets from 3 individuals with HLA-A2 or HLA-B7. The patient's sera also lost the reactivity with these RBCs after treatment with chloroquine. Therefore, it is likely that unidentified RBC reactive antibodies were RBC cross reactive HLA antibodies (i.e., anti-Bg).

DTT-treatment of her serum day 21 was also performed to distinguish between primary or secondary immune response. There was no influence by DTT-treatment against anti-E titer (64 by PEG-IAT) and anti-HLA-A2 titer (128 by LCT and 5120 by AHG-LCT) with non-treated and DTT-treated serum[10].

The MMA was performed using serum obtained at day 578. The patient's serum showed 3.7% (22/600) reactivity against Bg(a+) RBCs, compared to 0.2% (1/600) against Bg(a-) compatible RBCs. And Bg(a+) cells

showed 0% (0/600) with a negative control serum and 10.3% (62/600) with a serum containing strong anti-HLA.

Unexpectedly, in serum at day 578 no anti-E, anti-c, or anti-Jk^a was detectable whereas anti-HLA was detected, although the titer was decreased (e.g., a titer of 4 against HLA-A2 by LCT and a titer of 256 by AHG-LCT).

To investigate whether the patient had been sensitized to these antigens by pregnancy, we tested her son's RBC antigens and HLA type. He was incompatible only with Jk^a antigen. His RBC had the same E and c antigens with the patient's, and his HLA type, homozygotes of haplotype A33 and B52, was common with the patient's (Table 1).

Discussion

HTR due to HLA antibodies have previously been reported[4-7,11,12] . Although HLA antibodies are usually benign, HLA incompatibility may result in HTRs or decreased RBC survival when recipients have strong antibodies to HLA antigens such as Bg^a (B7), Bg^b (B17) and Bg^c (A28/A2) that are strongly expressed on RBCs. Arndt and Garratty[9] reported interesting results showing that a single example of anti-Bg^a gave a positive MMA results (61% reactivity) against pooled commercial Bg(a+) red cells as targets suggesting potential clinical significance.

In our case, the first DHTR was induced by RBC antibodies, however, we could not identify which of the three antibodies was mainly responsible for the hemolysis. Benson et al.[7] described a HTR associated with HLA antibodies where the routine crossmatch was compatible but microscopic incompatibility was observed. In our case, Donor 4 was crossmatch compatible although the donor has HLA-A2. This might be due to too small amounts of Bg^c antigen on the RBCs of Donor 4 to detect by routine crossmatch test. We cannot exclude the possibility of detecting incompatibility if microscopic examination was used for crossmatch.

It is not easy to identify Bg antibodies correctly. First, Bg antigens are expressed to variable degrees on RBCs[13,14]. Second, there are only a few commercial panel cells with Bg antigen designation. Third, expression of Bg antigens possibly decreases during storage. Fourth, HLA antibodies when produced are often multispecific.

We thought that Bg antibodies could be identified by the following

examinations: 1) detection of the HLA antibody with the specificity of corresponding anti-Bg in the patient's serum; 2) loosing the reactivity by destruction of Bg antigens (HLA class I antigens) by chloroquine diphosphate[8] or solution of glycine-HCl/EDTA[15]; and 3) absorption of HLA antibodies in patient's serum with pooled platelets.

In this case, multireactive HLA antibodies (strongly reactive with HLA-A2 and -B7) detected in her serum after transfusion, did not react with RBCs treated by chloroquine, and the reactivity was eliminated by absorption with pooled platelets. Therefore, it is likely that the unidentified antibodies were RBC cross-reactive HLA antibodies (i.e., anti-Bg^{a}+Bg^{c}).

HLA antibodies are often found in multitransfused patients and multiparous women, however, HTRs are rarely encountered. Thus, it may be due to the rarity of the combination of a high titer HLA antibody in recipients and enough Bg antigen expression on transfused RBCs. The amount of Bg antigens on RBCs varies between individuals and among RBCs even in an individual[16]. And, the antigens may decrease by preservation[17]. The RBCs transfused of the case were relatively fresh (all 5 – 8 days of age) and this may have contributed to the hemolysis. However, there is no evidence whether irradiated RBCs might be more susceptible to HLA antibody induced lysis.

In the study of anti-HLA-B7 and its CREG (i.e., anti-Bg^{a}) by van der Hart[18], the ^{51}Cr study showed a two-component curve with t_{50} of about 100 min for some cells; the rest of the cells had a t_{50} of 20 hours. Nordhagen[4] also showed a two-component curve for the study of anti-HLA-A28 (i.e.,

anti-Bg^{c}), but different from the study of van der Hart in time with a small component showing $t_{1/2}$ of 1.5 days and a larger component showing 22 days survival. Our case fitted mostly with the study of van der Hart: acute hemolysis soon after transfusion of RBCs of Donor 4 and hemoglobinuria (possibly later reaction) on the next two days. The cause of a two-component curve in survival is an acute complement-mediated intravascular destruction and probably extravascular for late destruction. And, it is speculated that in some donors there is variable HLA expression on red cells, with some cells having much lower levels[16].

The percentage reactivity (3.7%) for MMA in our patient, was not high enough to satisfy the clinical significance for an acute HTR[9], as a cutoff of 3% reactivity has been used for many years to determine a positive versus negative assay. However, the low reactivity could be due to weakened activity in the serum sample 1.5 years after transfusion. The 3 RBC antibodies detected originally as a cause of the DHTR also had dropped below the serological detection level in the serum 1.5 years after the DHTR.

The patient had no previous transfusion history but had one pregnancy, in which the family study did not reveal the child's cells being the immunogen for sensitization of anti-E, anti-c, or anti-HLA of the patient. However, these antibodies were likely produced by an anamnestic immune response to the transfusion, which was suggested with the observation of anti-E and anti-HLA exclusively of IgG class evidenced by DTT treatment of her serum. We assume that the history was poor and that she may have been transfused (sensitized) during her surgery for ovarian cyst rather than

there being a primary response.

In conclusion, it is necessary to consider the contribution of HLA antibodies reactivity in cases where there is evidence of a HTR with no other RBC antibodies detected.

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Legends for figure

Figure 1

Clinical course of the patient. Two episodes of hemolytic transfusion reactions were observed; the first was delayed type occurred between days 12 and 13, and the second was acute type between days 22 and 24 (occurred 4 hours after the beginning of RBCs transfusion of Donor 4).

■ indicates hemoglobinuria, ▭ indicates fever

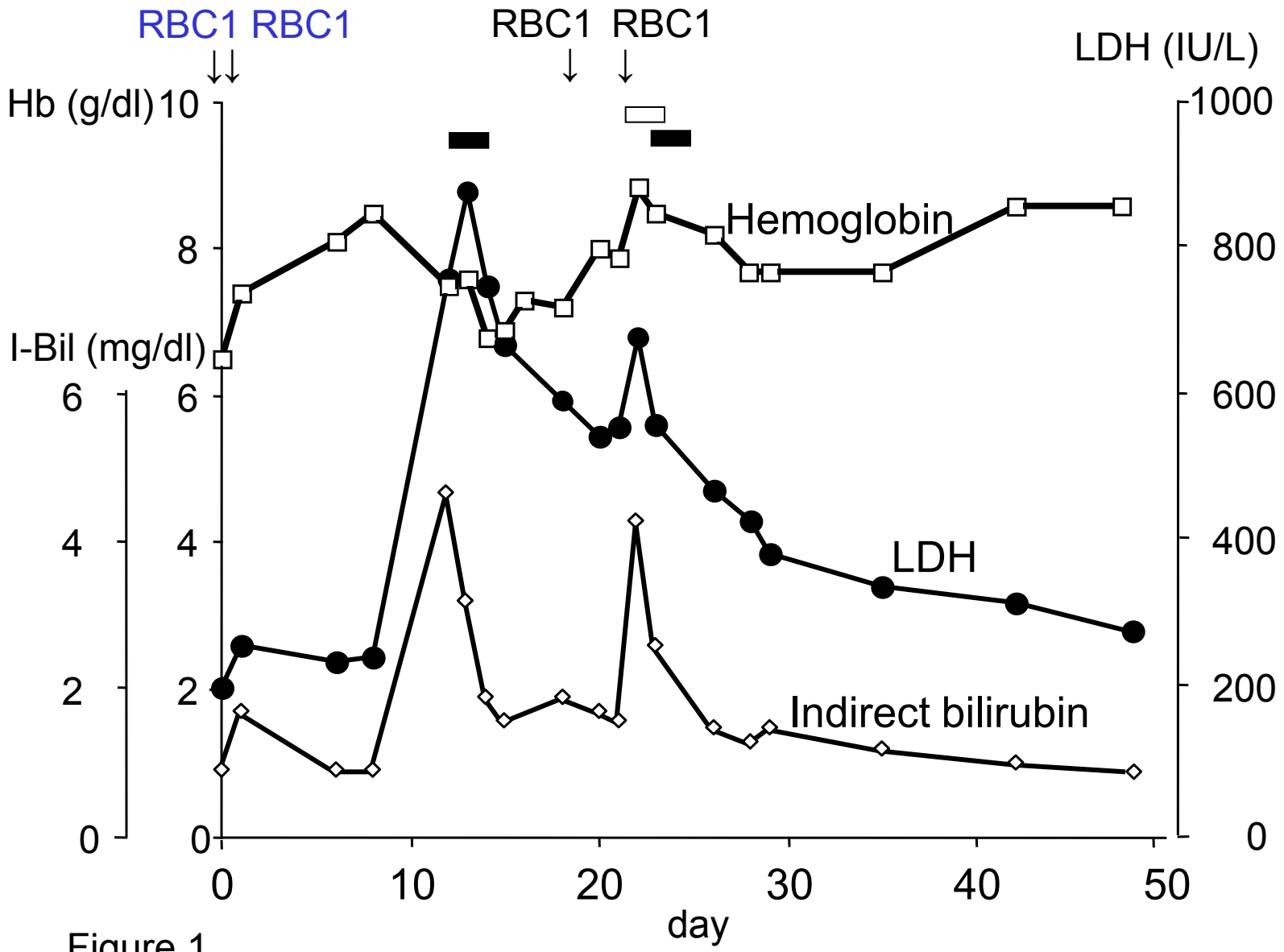
RBC1 RBC1
RBC1 RBC1
LDH (IU/L)
Hb (g/dl)
I-Bil (mg/dl)
Hemoglobin
LDH
Indirect bilirubin
day
0
10
20
30
40
50
2
4
6
8
10
200
400
600
800
1000

Figure 1

Table1. RBC antigens and HLA type of the patient and of RBC donors

Donor	Age (days)	Transfusion day	Rh	Jk	HLA
1	5	day 0	D+C+c+E+e+	a-b+	A2*, - B46,61**
2	5	day 1	D+C+c- E-e+	a+b+	A2*,31 B61**, -
3	7	day 19	D+C+c- E-e+	a-b+	A24, - B52,48**
4	8	day 22	D+C+c- E-e+	a-b+	A2*,26 B35,54
Patient			D+C+c- E-e+	a-b+	A26,33 B52,62
Patient's son			D+C+c- E-e+	a+b+	A33,- B52, -

* Corresponding with Bg^c

* * Crossreactive with HLA-B7 and HLA-B7/61/48 correspond to Bg^a