Prevalence and clinical significances of red cell alloimmunization and red cell bound immunoglobulin G in polytransfused patients with thalassemias

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ABSTRACT

The study was to determine the prevalence and clinical significances of red blood cell (RBC)-bound IgG as detected by flow cytometry in polytransfused patients with thalassemias. Relationship of the presence of RBC-bound IgG with RBC alloimmunization was also evaluated. This study included 59 polytransfused patients with β-thalassemia disease. We studied the frequency of RBC autoantibodies and alloimmunization. Direct Coombs test and flow cytometry were performed to detect the presence of RBC autoantibodies while RBC alloantibodies were detected by antibody screening and identification assays.

Eight (13.6%) and 34 (57.6%) patients were found a positive direct Coombs test and flow cytometry, respectively. Twenty (33.9%) patients developed RBC alloantibodies. The four most frequent RBC alloantibodies were anti-E (55%), anti-Mia (40%), anti-Di(a) (25%) and anti-Cc (15%), respectively. There was no significant difference in the presence of RBC-bound IgG between polytransfused with thalassemia patients who developed RBC alloimmunization (13 of 20; 65%) and those without RBC alloantibodies (21 of 39; 53.8%), p = 0.412. Splenectomy was significantly associated with the presence of RBC-bound IgG but not with RBC alloantibody formation.

The overall frequency of RBC alloantibody formation in polytransfused patients with thalassemias was 33.9%. The most common RBC alloantibody was anti-E. RBC autoantibody formation was more frequently detected by flow cytometry (57.6%) than by direct Coombs test (13.6%). Splenectomy was significantly associated with the development of autoreactive RBC-bound IgG antibodies in the polytransfused patients with thalassemias. The presence of the anti-RBC autoantibodies may cause an increase of transfusion requirement.

Introduction

Thalassemias are a heterogenous group of hereditary anemias caused by gene mutations affecting the production of α- or β-globin [1]. Anemia is a consistent feature of the thalassemia syndromes, resulting from ineffective erythropoiesis and hemolysis, both of which are a consequence of unbalanced globin chain synthesis. The regular red cell transfusion is the main supportive choice for severe forms of thalassemias. One of the major consequences of transfusion is the formation of alloantibodies and in some patients autoantibodies against red blood cell (RBC) antigens [2]. Alloimmunization to RBC antigens is an immune response often triggered by transfusion of RBCs and pregnancy. After transfusions, IgG antibodies against donors’ RBCs may be formed. These IgG antibodies bind to and may cause destruction of the transfused RBCs [3]. Several studies have reported variable frequencies of RBC alloimmunization in patients with thalassemias [4–6]. Mechanism of RBC alloimmunization is complex but involves at least three main contributing factors which include the RBC antigenic difference between the blood donor and the recipient, the recipient’s immune status and the immunomodulatory effect of blood transfusion on the recipient’s immune system [5]. In addition, splenectomized patients with thalassemias may have a higher RBC alloimmunization rate. The absence of a spleen may expedite the immune response to the infused foreign antigens which are not effectively filtered [7].

Erythrocyte autoantibodies, although less frequently detected in thalassemias, may result in clinical hemolysis and difficulty in cross-matching of blood [8]. Formation of RBC autoantibodies may also render higher transfusion requirement due to accelerated destruction of both patients’ own as well as the transfused red cells [9]. Such patients may require immunosuppressive therapy such as corticosteroids to alleviate the severity of anemia [5].

Detection of immunoglobulin G (IgG) and complement bound to RBCs by direct Coombs test has been considered as the gold standard test in the diagnosis of immune hemolysis [10]. However, this test is...
not sensitive enough to detect less than 500 IgG molecules per red cell. In this study, flow cytometry has been applied to detect RBC-bound IgG due to its high accuracy, reproducibility and sensitivity [11–13]. Furthermore, it can also be potentially applied for quantitative determination of immunoglobulin molecules expressed on cell surface using an external standard, such as quantum simply cellular (QSC) beads [12]. The objective of the study was to determine the prevalence and clinical significances of RBC-bound IgG as detected by flow cytometry in polytransfused patients with thalassemias. Relationship of the presence of RBC-bound IgG with RBC alloimmunization was also evaluated.

Materials and methods

Patients and normal controls

Fifty-nine polytransfused patients with β-thalassemia disease with age more than 17 years who followed at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, were included in the study. Each of them received regular transfusion at an interval of 3–5 weeks. There were two patients whose data of initial transfusions at other hospitals were not available. Increased transfusion requirement was defined as an apparent increase of units of blood transfused per month within 12 months of the study. This study was approved by the Faculty Institutional Review Board. Informed consents were obtained from the patients and 30 normal controls before blood samples were collected in ethylene-diaminetetraacetate (EDTA). The diagnosis of thalassemia was based on a standard hemoglobin analysis, mostly with high-performance liquid chromatography (HPLC). The concentration of hemoglobin in each patient was determined by Sysmex XE-5000 automatic hematology analyzer.

Red blood cell alloimmunization, antibody screening and identification

Erythrocyte alloantibodies were studied in patients’ sera using standard blood bank methods. ABO and Rh blood grouping was done by the standard tube method for all patients. Screening test and antibody identification test were performed using gel cards and commercial RBC panel tube test, respectively to detect alloimmune antibodies formed against RBC antigens in the sera. The antibody identification was performed only when the antibody screening was positive.

Detection of red blood cell bound antibody

Detection of RBC bound IgG and/or complement by direct Coombs test was performed using polyclonal antihuman globulin reagent in standard glass tube according to the standard protocol [14]. RBC-bound IgG was also detected by flow cytometry, briefly, EDTA blood was centrifuged at 1500 rpm for 10 minutes. Packed red cells were washed and resuspended in PBS. RBC suspension was incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG (AbD Serotec, Oxford, UK) at room temperature for 60 minutes in the dark. In the isotype (negative) control tube, RBCs were incubated with FITC-conjugated mouse IgG1 (AbD Serotec, Oxford, UK). The cells were washed with PBS and suspended in 1% paraformaldehyde solution. Finally, the RBCs were analyzed by a flow cytometry (FACS Calibur, Becton and Dickinson, U.S.A.). As an internal process control of the assay method, a known positive and negative (normal donor) control samples were used to run with each experiment of patient samples.

Quantitation of IgG molecules

Quantitative analysis of RBC-bound IgG was performed using QSC beads (Bangs Laboratories Inc., Fisher, IN, U.S.A.). The QSC beads consist of four bead populations of the same size coated with goat anti-mouse IgG (Fc-specific) antibodies which have differing capacities to bind mouse monoclonal IgG, and one blank bead population with no specific binding capacity for mouse IgG. Briefly, each combination of the four-level bead populations in a single tube was incubated with FITC-conjugated mouse anti-human IgG at room temperature for 60 minutes in the dark. The tube with blank bead population was run first and followed by all the other tubes with antibody-coated beads. A calibration curve was established by plotting the acquired fluorescent intensities from the QSC beads, expressed in geometric mean area, against the manufacturer-assigned antibody-binding capacity values (or IgG molecules) using Excel spreadsheet template-QuickCal software provided by Bangs Laboratories. Using the same instrument setting, the number of IgG molecules per RBC of unknown samples was then determined by comparing its fluorescent intensity against the calibration curve.

Interpretation of results

Results of flow cytometry were expressed as mean fluorescent intensity (MFI). MFI value was calculated as geometric mean of each tested sample after subtraction by geometric mean of isotype (negative) control. Furthermore, quantitation of IgG molecules was calculated as the number of IgG molecules after subtraction by the number of IgG molecules of isotype control.
Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS) software. Data were presented as number and frequency (%). Chi-square test or Fisher’s exact test was applied to compare categorical variables. Mann–Whitney U test, independent samples t-test or paired samples t-test was used to compare data between two groups. The level of agreement between positive direct Coombs test and flow cytometry was evaluated by the κ coefficient. The interpretation of κ coefficient is as follow: less than 0 = less than chance agreement, 0.01–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, 0.81–1.00 = almost perfect agreement. p-Value of ≤0.05 was considered statistically significant.

Results

Among the 59 polytransfused patients with thalassemias, 5 had β-thalassemia major ( homozygous β-thalassemia) and 54 had HbE/β-thalassemia. They were 18 (30.5%) males and 41 (69.5%) females. The age range and mean age were 17–64 and 32 years, respectively. The previous period of transfusion ranged from 2 to 32 years (Table 1). Fifty out of 57 (87.7%) patients started their first transfusions since childhood.

Frequency, specificity and relationship of red blood cell alloimmunization and RBC-bound IgG

Twenty patients (33.9%) were found to have RBC alloantibodies. The prevalence of RBC alloimmunization seemed to be higher in females (17/41; 41.5%) than in males (3/18; 16.7%), but without statistical significance (p = 0.064). RBC alloimmunization was not influenced by age of starting transfusion, period and total units of previous blood transfusion (data not shown). The frequencies of RBC alloimmunization among non-splenectomized (13/39; 33.3%) and splenectomized (7/20; 35%) patients were not different (Table 2).

Among the patients with red blood cell immunization, nine RBC alloantibody specificities were identified. The four most frequent RBC alloantibodies were anti-E (55%), anti-Mia (40%), anti-D(a) (25%) and anti-C (15%), respectively (Table 3). Single and multiple antibody specificities were found in 7 (35%) and 13 (65%) patients, respectively. The majority of patients with both RBC alloantibodies and RBC-bound IgG (71.4%) and patients with RBC alloantibodies only (83.3%) had multiple antibodies of which anti-E was the most common RBC alloantibody detected followed by the anti-Mia (Table 3).

In this study, there was no significant difference in the presence of RBC-bound IgG between patients

| Table 1. Clinical data of polytransfused patients with thalassemias. |
|----------------------------------|-------|
| Demographic data | n (%) |
| Thalassemia diagnosis | 59 |
| β-thalassemia major | 5 (8.5) |
| HbE/β-thalassemia | 54 (91.5) |
| Mean age: range (years) | 32: 17–64 |
| Gender | |
| Males | 18 (30.5) |
| Females | 41 (69.5) |
| M: F | 1: 2.3 |
| Age at first transfusion; mean: range (years) | 7: 0.3–47 (n = 57) |
| Period of transfusions; mean: range (years) | 19: 2–32 (n = 57) |
| Splenectomy | |
| Yes | 20 (33.9) |
| No | 39 (66.1) |
Figure 1. Box plot represents mean ± SD of MFI values (A) and number of IgG molecules (B) in polytransfused patients with thalassemias.

Note: *P*-value from Mann–Whitney *U* test, *p* < 0.05 statistically significant; (+) = positive; (−) = negative; FC (IgG) = RBC-bound IgG as detected by flow cytometry.

Table 2. Data of patients with RBC alloantibodies and RBC-bound IgG.

| Presence of alloantibodies | Non-alloantibodies | Presence of RBC-bound IgG | No-RBC bound IgG |
|---------------------------|--------------------|---------------------------|-------------------|
| Presence of alloantibodies n (%) | Non-alloantibodies n (%) | Presence of RBC-bound IgG n (%) | No-RBC bound IgG n (%) |
| Total patients | 20 (33.9) | 39 (66.1) | 34 (57.6) | 25 (42.4) |
| Thalassemia diagnosis | | | | |
| Beta-thalassemia major | 2 (40) | 3 (60) | 3 (60) | 2 (40) |
| HbE/β-thalassemia | 18 (33.3) | 36 (66.7) | 31 (57.4) | 23 (42.6) |
| Mean age; range (years) | 35: 17–61 | 31: 19–64 | 31: 17–61 | 33: 19–64 |
| Gender | | | | |
| Males | 3 (16.7) | 15 (83.3) | 11 (61.1) | 7 (38.9) |
| Females | 17 (41.5) | 24 (58.5) | 23 (56.1) | 18 (43.9) |
| Age at first transfusion; mean: range (years) | 8: 0.3–42 (n = 19) | 7: 0.3–47 (n = 38) | 5: 0.3–20 (n = 33) | 10: 0.5–47 (n = 24) |
| Period of transfusions; mean: range (years) | 20: 3–32 (n = 19) | 18: 2–30 (n = 38) | 20: 3–32 (n = 33) | 16: 2–32 (n = 24) |
| Spleenectomy | | | | |
| Yes | 7 (35) | 13 (65) | 16 (80) | 4 (20) |
| No | 13 (33.3) | 26 (66.7) | 18 (46.2) | 21 (53.8) |

Table 3. The distribution frequency of RBC alloantibodies in the 20 thalassemia patients with positive antibody screen.

| Alloantibody specificities | Total Alloantibodies (n = 20) | Single antibody specificity (n = 6) | Multiple antibody specificities (n = 7) | Total (n = 13) | Single antibody specificity (n = 1) | Multiple antibody specificities (n = 6) | Total (n = 7) |
|---------------------------|-------------------------------|-----------------------------------|----------------------------------------|----------------|-----------------------------------|----------------------------------------|----------------|
| Anti-E                    | 11 (55%)                      | 1                                 | 5                                      | 6 (30%)        | -                                 | 5                                      | 5 (25%)        |
| Anti-Mia                  | 8 (40%)                       | 2                                 | 3                                      | 5 (25%)        | 3                                 | 2                                      | 3 (15%)        |
| Anti-Di(a)                | 5 (25%)                       | 1                                 | 2                                      | 3 (15%)        | 2                                 | 2                                      | 2 (10%)        |
| Anti-c                    | 3 (15%)                       | -                                 | 1                                      | 1 (5%)         | 2                                 | 2                                      | 2 (10%)        |
| Anti-Le(a)                | 2 (10%)                       | -                                 | 1                                      | 1 (5%)         | 1                                 | 1                                      | 1 (5%)         |
| Anti-Le(b)                | 2 (10%)                       | -                                 | 1                                      | 1 (5%)         | 1                                 | 1                                      | 1 (5%)         |
| Anti-P1                   | 2 (10%)                       | -                                 | 2                                      | 2 (10%)        | -                                 | -                                      | -              |
| Anti-S                    | 2 (10%)                       | -                                 | 1                                      | 1 (5%)         | -                                 | 2                                      | 2 (10%)        |
| Anti-M                    | 1 (5%)                        | -                                 | 1                                      | 1 (5%)         | -                                 | -                                      | -              |
| Unidentified Ab           | 9 (45%)                       | 1                                 | 4                                      | 5 (25%)        | 1                                 | 3                                      | 4 (20%)        |
who developed RBC alloimmunization (13 of 20; 65%) and those without RBC alloantibodies (21 of 39; 53.8%), \( p = 0.412 \). Similarly, there was no difference in the presence of RBC-bound IgG between patients with single RBC alloantibody (6/7; 85.7%) and those with multiple RBC alloantibodies (7/13; 53.8%), \( p = 0.177 \).

### Clinical significances of RBC-bound IgG

Thirty-four (57.6%) of 59 patients were found to have RBC-bound IgG as detected by flow cytometry. There were no statistically significant differences in gender, age at first transfusion and previous period of transfusions between thalassemia patients who had and had no RBC-bound IgG. RBC-bound IgG was more prevalent among splenectomized patients (16/20; 80%) than non-splenectomized patients (18/39; 46.2%) (\( p = 0.013 \); Table 2). Presence of RBC-bound IgG did not significantly increase transfusion requirement (Table 4). However, among the 59 polytransfused patients with thalassemias, 13 patients had increased evidence of transfusion requirement as shown by average units of blood transfused per month or RBC consumption during 12 months after the study when compared with 12 months before the study (Table 5). The mean RBC consumption of these 13 patients during 12 months after the study was significantly higher than before the study (121 ± 59 vs. 93 ± 61; \( p < 0.01 \)). Recent increase of transfusion requirement was more frequent among patients with positive flow cytometry than those without RBC-bound IgG (11/34; 32.4% vs. 2/25; 8%, \( p = 0.026 \)) and also more frequent in splenectomized patients than non-splenectomized patients (8/20; 40% vs. 5/39; 12.8%, \( p = 0.024 \)).

### Discussion

In our study, the sensitivity of flow cytometry for detection of RBC-bound IgG was shown to be higher than the direct Coombs test in polytransfused patients with thalassemias (57.6% vs. 13.6%). The level of agreement between positive direct Coombs test and flow cytometry for detection RBC-bound IgG in polytransfused patients with thalassemias was fair. While the direct Coombs test effectively diagnoses autoimmune hemolytic anemia (AIHA) when more than 500 IgG molecules are bound to the red cell, flow cytometry, a highly sensitive antibody detection technique, can detect as low as 30–40 IgG molecules per red cell [15]. Variable rates of RBC alloimmunization ranging from 3.1% to 50% were observed in transfusion-dependent thalassemia patients of different ethnic origins [8,16]. The higher RBC alloimmunization rate was probably due to the heterogeneity of the populations with mismatched RBC phenotypes between donors and recipients [3,17]. In our polytransfused patients with thalassemias, the RBC alloimmunization rate was 33.9% which may be explained by heterogeneity between the red cell antigens of recipients and donors. The most common RBC antibodies associated with alloimmunization in our study were anti-E (55%) and anti-Mia (40%). This is similar to the finding by Cheng et al. [18] who found that anti-E (39.3%) was the most frequent RBC alloantibody followed by anti-Mia (30.85%) in thalassemia major patients with chronic blood transfusion. Anti-E was found to be the most prevalent RBC alloantibody among transfusion-dependent thalassemia patients in Asian population [5,18]. While anti-Mia is a common RBC alloantibody in Thais, Chinese and Taiwanese, it is rarely found in Caucasians [19].

Our study, as well as the others [3,20], did not suggest gender as a predisposing factor to the development of RBC alloimmunization. On the other hand, an association between RBC alloimmunization and gender had been reported with controversial results, either a higher risk of RBC alloimmunization in female [21] or in male patients [22]. Our study did not demonstrate associations of age at first transfusion and period

### Table 4. Comparing the mean RBC consumption before and after the study in polytransfused patients with thalassemias.

| Group                      | Mean RBC consumption (ml/kg/year) | 12 months before the study | 12 months after the study | p-value |
|----------------------------|-----------------------------------|-----------------------------|---------------------------|---------|
| DCT + FC positive (n = 8)  | 71                                | 70                          | 66                        | 0.062   |
| FC positive only (n = 26)  | 142                               | 147                         | 0.237                     |         |
| DCT + FC negative (n = 25) | 106                               | 103                         | 0.162                     |         |

DCT + FC positive = Presence of RBC-bound IgG as detected both by direct Coombs test and flow cytometry.

FC positive only = Presence of RBC-bound IgG as detected by flow cytometry only.

DCT + FC negative = Absence of RBC-bound IgG as detected both by direct Coombs test and flow cytometry.

### Table 5. Data of average units of blood transfusion and RBC consumption before and after the study in 13 polytransfused patients with thalassemias who had increased transfusion requirements.

| No. | Average units of blood transfusion (units/month) | Average RBC consumption (ml/kg/year) | Average units of blood transfusion (units/month) | Average RBC consumption (ml/kg/year) |
|-----|--------------------------------------------------|-------------------------------------|--------------------------------------------------|-------------------------------------|
| 1   | 1.5                                              | 70                                  | 2                                                | 81                                  |
| 2   | 2                                                | 207                                 | 3                                                | 224                                 |
| 3   | 1                                                | 62                                  | 2                                                | 109                                 |
| 4   | 1                                                | 52                                  | 2                                                | 99                                  |
| 5   | 2                                                | 204                                 | 3                                                | 230                                 |
| 6   | 1                                                | 52                                  | 2                                                | 73                                  |
| 7   | 1                                                | 135                                 | 2                                                | 182                                 |
| 8   | 1                                                | 63                                  | 2                                                | 98                                  |
| 9   | 1.5                                              | 54                                  | 2                                                | 78                                  |
| 10  | 1                                                | 118                                 | 2                                                | 143                                 |
| 11  | 1                                                | 55                                  | 2                                                | 82                                  |
| 12  | 1                                                | 49                                  | 1.5                                              | 65                                  |
| 13  | 1                                                | 108                                 | 2                                                | 134                                 |
of transfusions with the development of either RBC alloimmunization or RBC-bound IgG in polytransfused patients with thalassemias. Despite not performing elution absorption test, RBC-bound IgG in our patients were likely autoantibodies rather than alloantibodies coated on residual donor cells. In \( \beta \)-thalassemic red cells, excess \( \alpha \)-globin containing hemes, through the oxidative process, tend to bind and alter membrane proteins leading to a formation of neoantigens with subsequent induction of anti-RBC autoantibodies \([5,23,24]\). Singer et al. \([5]\) found RBC autoimmunization in 16 of 64 (25\%) transfusion-dependent thalassemia patients of Asian descent, 68.8\% of them had IgG warm autoantibodies. Cheng et al. \([18]\) observed warm reactive RBC autoantibodies in 18 out of 88 (20.5\%) patients with thalassemia requiring chronic transfusion, 11 of them (61.1\%) had autoantibodies without alloantibodies. Ameen et al. \([3]\) reported that 20 of 21 (95\%) transfusion-dependent thalassemia patients who developed RBC alloantibodies also had associated RBC autoantibodies, as proven by elution technique. It was suggested that the alloantibody may also drive autoantibody formation. The previous study in multitransfused patients with thalassemia major showed a significant B-lymphocytosis consistent with ongoing B-cell stimulation associated with chronic exposure to transfused red-cell antigens \([25]\). B lymphocyte changes may reflect the effect of cytokines produced during blood storage \([26]\) together with stimulation by minor incompatibility red cell antigens. This B-cell stimulation is accompanied by an increase in serum immunoglobulins, immune complexes, and cells expressing surface immunoglobulin. These immune alterations in thalassemia patients may explain an increased chance of RBC autoantibody formation in the presence of alloimmunization \([27]\). As our study was carried on blood samples from the patients, at least three weeks after previous transfusions, alloantibody-bound transfused RBCs were likely to be removed and not detected in the circulation during the study.

In this study, we found that splenectomized patients with thalassemias were more likely to have RBC autoantibodies but not RBC alloantibodies. This finding was consistent with the results from other studies \([8,28]\). Furthermore, several studies had reported no significant association between splenectomy and the development of RBC alloantibodies \([4,29,30]\). On the other hand, Ahmed et al. \([2]\) and Thompson et al. \([31]\) reported a significant association of splenectomy in thalassemia patients with both RBC alloantibodies and autoantibodies. However, Singer et al. \([5]\) observed that thalassemia patients who had been splenectomized acquired a higher RBC alloimmunization rate than those with non-splenectomized patients. These conflicting results may involve various factors including RBC transfusion burden, timing of initial RBC antigen exposure (pre-splenectomy vs. post-splenectomy), and the life span of transfused RBCs \([32]\). In the absence of an efficient filtering organ following splenectomy, more thalassemic RBCs with deposition of opsonizing autologous IgG are left in circulation as observed in our studied patients.

In the present study, there was an association between increased transfusion requirements with the formation of RBC autoantibody. It is possible that deposition of opsonizing autologous immunoglobulins and possibly also complement C3 fragments \([33]\), increases phagocytic removal of the thalassemia erythrocytes resulting in increased requirement for transfusions. In agreement with our study, Dhawan et al. \([8]\) found that the development of RBC autoantibody was significantly higher in patients receiving more transfusions.

In summary, the overall frequency of RBC alloantibody formation in polytransfused patients with thalassemias was 33.9\%. The most common RBC alloantibody was anti-E. RBC autoantibody formation was more frequently detected by flow cytometry (57.6\%) than by direct Coombs test (13.6\%). Splenectomy was significantly associated with the development of autoreactive RBC-bound IgG antibodies in the polytransfused patients with thalassemias. The presence of the anti-RBC autoantibodies may cause an increase of transfusion requirement.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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