Erythrocyte autoantibodies and expression of CD59 on the surface of red blood cells of polytransfused patients with ß-thalassaemia major

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ABSTRACT

Repeated transfusions for the treatment of thalassaemia major cause an insult to the patient's immune system and provoke post-transfusion purpura and haemolytic reactions that can be severe and life threatening. This study aims to investigate the presence of erythrocyte autoantibodies and CD59 expression on the surface of red blood cells (RBCs) in patients with ß-thalassaemia major, and any relationship to frequency of blood transfusion. The study looks at a total of 49 patients (both children and adults) with ß-thalassaemia major, divided into four groups according to the number of blood transfusions received and the presence or absence of the spleen. Glycosylated haemoglobin, Coombs' test (direct and indirect) and CD59 level on the RBC surface (by flow cytometry) are estimated in all patients studied. Glycosylated haemoglobin level was significantly lower in those who had received less than 10 units of blood (group III) than in those who had received more than 25 units of blood and had undergone splenectomy (group Ib), and was significantly lower in those who had received 10–25 units of blood (group II) than in those that comprised group Ib ($F=3.598$, $P=0.0205$). Considering CD59 expression, there was a marked difference between the groups. Expression was highest in group III and diminished progressively through groups II, Ia (polytransfused, non-splenectomised) and Ib ($F=19.83$, $P=0.0000$). No relationship was observed between CD59 expression and either blood group or gender. A significant negative correlation between CD59 expression and reticulocyte percentage ($r=–0.538$, $P=0.000$) and normoblast count ($r=–0.5455$, $P=0.000$) was found. A negative correlation between lymphocytosis and CD59 expression was also noted in groups III ($r=–0.745$, $P=0.013$), Ia ($r=–0.5849$, $P=0.022$) and Ib ($r=–0.6711$, $P=0.009$). Direct Coombs' test was positive in only one patient in group Ib, who also showed the lowest haemoglobin level. Thalassaemia patients exposed to multiple antigens through repeated blood transfusions showed lower CD59 expression than did those who had received fewer transfused units, which is a good method of detecting potential autoantibodies. Furthermore, a negative Coombs' test does not exclude autoimmunisation in such patients.

KEY WORDS: Antigens, CD59. Autoantibodies. beta-Thalassemia. Coombs' Test. Hemoglobin A, glycosylated.

Introduction

Although blood transfusions are common and essential in the management of thalassaemia patients, there are numerous risks and considerable morbidity associated with long-term transfusion therapy. Repeated exposure can induce alloimmunisation to erythrocyte antigens, leading to difficulty in finding compatible blood.[1] The development of erythrocyte autoantibodies as a result of repeated transfusion is less commonly recognised and has been described only in case reports or small series. In addition, the autoantibodies were associated with clinically significant haemolysis in some of these patients.[1,2]

However, erythrocyte alloimmunisation is not necessary for autoantibody formation as certain people may have a genetically increased tendency to develop erythrocyte autoantibodies. Moreover, the effect of splenectomy and subsequent immune dysregulation cannot be underestimated in the development of autoantibodies.[1]

An alternative explanation is that the intravascular environment in patients with haematological disorders increases the opportunity for erythrocyte autoantibody formation. Repeated deformation of the erythrocyte membrane and abnormal interactions with the endothelium may lead to exposure of the erythrocyte neoantigens that induce IgG autoantibody formation.[1]

Exposure of inner leaflet phospholipid components also can lead to deposition of surface complement via the activation of the alternative pathway.[3] Although a positive direct antiglobulin test is not always clinically significant, the presence of surface complement on autologous erythrocytes may be associated with clinically significant haemolysis.[4]

Human CD59 antigen (protectin) is an 18–25 kDa glycoprotein inhibitor of complement lysis. Protectin is widely expressed on human cells and tissues, and is present on all circulating blood cells, endothelial cells, most

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epithelial cells and on spermatozoa. Protectin is expressed on normal human myocardial cells but not on infarcted myocardium.[5]

A soluble form of protectin was first isolated from human urine. Subsequently, it has been found in saliva, tears, sweat, breast milk, blood plasma, amniotic fluid and seminal plasma.[6]

The function of protectin is to inhibit the final steps of membrane attack complex (MAC) assembly on cell membranes. Through binding to the C5b-8 complex, protectin limits C9 input and prevents formation of the polymeric C9 complex. Therefore, erythrocyte protectin inhibits the haemolytic activity of C5b-9 complexes.[7] The erythrocyte protectin binding sites on C8 and C9 have been localised to the C8 α-chain and C9b fragment. As yet, however, it is not clear which residues in protectin are critical for mediating its attachment to the MAC,[8] but it participates in T-cell rosette formation with erythrocytes, appears to be necessary for T-cell activation, and it is involved in antigen presentation.[9]

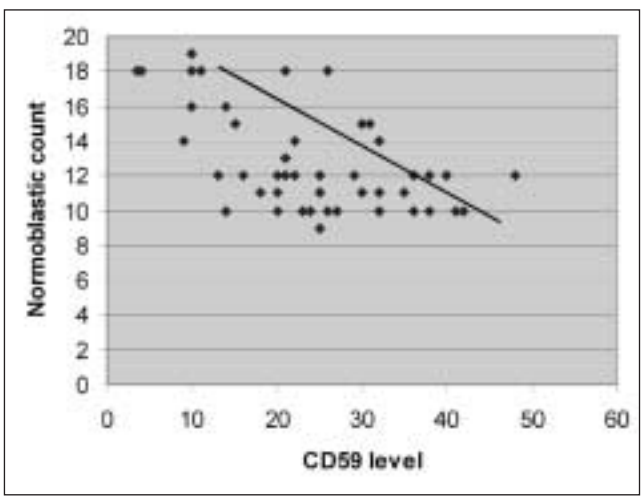
Although much research on, and reviews of, protectin have been published, little study of haematological disorders, especially of thalassaemia major, has been undertaken.

The aim of the present study is to investigate erythrocyte autoantibodies and CD59 expression on the surface of red cells in patients with β-thalassaemia, and any relationship with the frequency of blood transfusion.

Materials and methods

The present study was carried out on 49 children and adults previously diagnosed with β-thalassaemia major who were being followed up in the haematology clinic of Alexandria Children's Hospital at El-Shatby and at the Medical Research Institute Hospital in Alexandria. All children were receiving blood transfusions and most of them were undergoing desferrioxamine (Desferal) chelation. Patients with diabetes mellitus were excluded from the study. Study cases were classified into four groups according to the number of blood transfusions received and the presence or absence of a spleen.

Fig. 1. Correlation between CD59 expression and normoblast count.



Group Ia
The group comprised 15 patients (10 males, 5 females) who had received more than 25 units of blood and had not undergone splenectomy. Age ranged from four to 21 years (mean: 6.8 ± 4.3 years).

Group Ib
The group comprised 14 patients (6 males, 8 females) who had received more than 25 units of blood and had undergone splenectomy. Age ranged from 7–21 years (mean: 14 ± 4.9 years).

Group II
This group comprised 10 children (7 males, 3 females) who had received 10–25 units of blood. Age ranged from 2–3.5 years (mean: 2.83 ± 0.47 years).

Group III
This group comprised 10 children (6 males, 4 females) who had received less than 10 units of blood. Age ranged from 1–1.5 years (mean: 1.26 ± 0.24 years).

A detailed history was taken from all the patients in the study in order to ascertain the age at diagnosis, the period of time since first transfusion, the number and frequency of transfusions received, and the frequency and regularity of desferrioxamine chelation treatment. History of any episodes of increased haemolysis or increased need for blood transfusion was taken, and a complete physical examination was performed.

Laboratory investigations included complete blood picture and reticulocyte count, haemoglobin electrophoresis,[10] serum ferritin,[11] liver function tests (ALT, AST),[12] and Coombs' test (direct and indirect).[13] Glycosylated haemoglobin was assayed using a kit from Stanbio Laboratories, Texas, USA.[14] CD59 expression was assessed by flow cytometry[15] using mouse antihuman CD59 (RPE kit, Serotec, UK).

Heparinised venous blood was collected by venipuncture under aseptic conditions and tested without delay. Whole blood was washed in purified phosphate-buffered saline (PBS) and, after removal of the buffy coat, resuspended in PBS. Erythrocyte suspensions were stained by an indirect immunofluorescence technique using anti-CD59 monoclonal mouse antibody. A FACScan flow cytometer (forward and side scatter) was used to determine CD59 expression on at least 10,000 cells in each fraction.

Results

Table I summarises the laboratory data from the four groups studied. Fasting blood sugar was estimated to detect cases with high blood sugar as it causes elevation of glycosylated haemoglobin. It ranged from 70 to 150 mg/dL. No correlation was found between CD59 expression and blood group or gender.

Discussion

Although bone marrow transplantation has emerged as a cure for thalassaemia, blood transfusion and iron chelation

remain the mainstay of treatment. The factors associated with the development of alloimmunisation are complex and involve at least three main contributing elements: red cell antigenic differences between the donor and recipient, the recipient's immune status, and the immunomodulatory effect of the allogeneic blood transfusion on the recipient's immune system.[16]

The direct antiglobulin test is used to detect antibody bound to the patient's red cells that causes immune haemolysis. The findings presented here are in partial agreement with those of other researchers.

Singer *et al*. reported a positive Coombs' test in 16 (25%) out of 64 transfused patients with thalassaemia, causing severe haemolytic anaemia in three (18.75%) of 16 patients. Autoimmunisation was associated with alloimmunisation (especially to the Kell system) and with splenectomy in 44% and 56%, respectively. They reported that transfusion of phenotypically matched blood for the Rh and Kell systems (leucodepleted in 92%) compared to blood phenotypically matched for the standard ABO-D system (leucodepleted in 60%) showed significant effect in preventing alloimmunisation.[17]

Despite the recognition that autoantibodies are a transfusion-associated risk, little is known about the extent and causes of this phenomenon among thalassaemia patients and what preventive methods might be appropriate. Singer *et al*. suggested that erythrocyte conformational changes due to fragmentation, membrane deformability or alloantibody binding could result in the formation of autoantibodies, and that an altered deformability profile was more prevalent in splenectomised patients.[17]

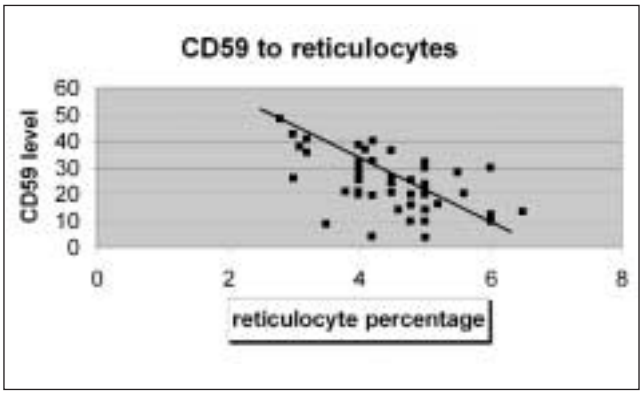
These findings are consistent with senescence of erythrocytes that may expose new antigens and promote or enhance an immune reaction, as known to occur in ageing and impaired red cells. This process is enhanced by the lack of an efficient system for the removal of damaged erythrocytes (i.e., removal of the spleen).[18]

Singer et al. attributed the high rate of alloimmunisation in their study of Asians living in North America to ethnic background, as the majority of their donor population was Caucasian.[17] Alloimmunisation rate in their study was higher than the 5% found in another study of transfused Caucasians.[19]

However, Ho and co-workers in Hong Kong have treated a comparable number of thalassaemia patients on regular transfusion with fully phenotypically matched blood to those who have an alloantibody in order to prevent further alloimmunisation.[20] They rarely needed to transfuse K-antigen-matched blood, and identified nine alloantibodies in five (7.4%) of their 68 patients. Only one of 68 had an autoantibody. Of the nine alloantibodies detected, only anti-E proved to be of clinical significance, causing a haemolytic transfusion reaction. All antibodies developed prior to the introduction of universal leucodepletion for the blood given to their thalassaemia patients. They explained their lower alloimmunisation rate to the use of phenotypically matched donors.

In the present study, a marked relative and absolute lymphocytosis, was observed in each group corrected to the corresponding age. This can be explained by the fact that removal of the spleen may result in an enhanced immune response to the infused foreign antigens, but may also be due to the effect of cytokines during blood storage, together with stimulation by minor incompatibility antigens.[21]

Fig. 2. Correlation between CD59 expression and reticulocyte count.



This view is supported by the findings of a number of studies.[21–24] Hodge *et al*.[22] observed that absolute lymphocytosis involved both T- and B-lymphocyte subsets, but that a greater increase occurred in the number of B cells. This was unrelated to the number of transfusions received, hepatitis B or C viral status, date of splenectomy, age or gender. Thus, it seems that splenectomy is not the sole determinant of lymphocytosis, as 38% of their patients who had not undergone splenectomy also showed lymphocytosis. This is in line with the findings of the study reported here.

In contrast, alloimmunisation and autoimmunisation were not frequent in our patients, perhaps due to immune tolerance because the patients often start transfusion therapy early in life. Almost all the patients included in the present study received their first transfusion when aged less than one. In this context, the work of Spanos and colleagues [25] supports the findings of the present study.

The immune response may also be affected by the number of blood units a patient receives. The relationship between the number of units transfused and antibody formation has not been investigated adequately in thalassaemia, but it is an important factor that contributes to increased alloimmunisation in patients, including those with sickle cell disease, who receive multiple transfusions.[19,26,27]

Autologous cells are protected against complement-mediated cell injury by the self-recognition mechanism using complement regulatory proteins composed of complement receptor type 1 (CR1, CD35), membrane co-factor protein (MCP, CD46), decay accelerating factor (DAF, CD55) and homologous restriction factor (protectin, CD59).[28] These glycolipid-anchored membrane proteins either induce C3 convertase dissociation (e.g., DAF) or prevent the full development of the membrane attack complex (e.g., CD59 or protectin).[9]

Decreased cell surface expression of CD59 renders erythrocytes more vulnerable to adherent C3 and C5 convertases and to polymerisation of C9 into membranes, and may enhance autologous complement-mediated lysis.[29] Decreased CD59 expression has been documented in paroxysmal nocturnal haemoglobinuria (PNH), as erythrocytes (type III) that lack CD59 and other phosphatidyl inositol-linked proteins are susceptible to lysis by the membrane attack complex. This knowledge has changed the methods of diagnosis for this disease and has

Table 1. Laboratory profile of the studied groups.

	Group Ia	Group Ib	Group II	Group III	F	*P*
Haemoglobin (g/dL)						
Mean ± SD	5.95±-1.2	6.3 ± 1.22	5.76±0.98	6.8±0.98		
Range	4.0–8.2	3.5–8	4.0–7.0	5–8	1.749	0.1704
Reticulocytes (%)						
Mean ± SD	4.7± 0.61	4.67 ± 1.02	4.58±0.65	3.69±0.6		
Range	3.8–6	3–6.5	4.0–6.0	2.8–4.5	4.418	0.0083*
Normoblasts (%)						
Mean ± SD	13.87± 2.92	13.57± 3.37	10.6±0.98	11.3±1.57		
Range	10–19	10–18	9.0–12.0	10–15	4.745	0.0058*
Serum ferritin (mg/dL)						
Mean ± SD	2279±924	2298±1285	1333±568	369±66		
Range	160–4000	178–4600	780–2500	290–500	11.99	0.0000*
Glycosylated haemoglobin (%)						
Mean ± SD	5.45±1	6.34±2.08	5.00±0.85	4.66±0.64		
Range	4–7.2	4.2–12	4.0–7.0	3.9–6	3.598	0.0205*
Lymphocytes (%)						
Mean ± SD	50.4 ± 9.65	51.71±10.16	64.9±3.7	66.7±6.27		
Range	40–78	35–64	60–70	59–67	19.83	0.0000*
CD 59 (%)						
Mean ± SD	22.87±8.38	17.35±10.47	25.87±5.64	36.13±6.36		
Range	9.8–40.4	3.5–41.2	20–36.4	25.8–48.7	13.35	0.0000*

*significant *(P<0.05)*

increased therapeutic options. Flow cytometric detection of CD59 is now applied as the sole diagnostic modality for PNH in many centres around the world. Recently, research has focused on CD59 transfer to deficient patients, as transfer of GPI-linked proteins from soluble preparations containing CD55 and CD59 to PNH erythrocytes has proved feasible and may have clinical utility.[30]

A decrease in CD59 reflects a higher susceptibility to complement-mediated lysis. In the present study, a profound decrease in CD59 expression was seen in one splenectomised female patient with marked lymphocytosis but a negative direct antiglobulin test, for whom it was difficult to find a matching blood unit.

Despite the fact that patients in groups Ia and Ib were more alloimmunised and showed lower levels of CD59 expression, active complement-mediated lysis was not evident. This indicates that small amounts of this regulatory protein are sufficient to prevent this type of cell lysis. This has been confirmed by Yuan *et al*. who showed that trypsin treatment of red blood cells destroys 80% of CD59 molecules but results in only slightly increased susceptibility to lysis. Thus, they concluded that partial CD59 activity is sufficient to protect cells from complement-mediated lysis.[31]

Low CD59 expression could be attributed to preferential loss via membrane vesicles shed from erythrocytes after calcium loading.[31] Alternatively, it has been demonstrated that thalassaemic erythrocytes carry C5b-9 complexes but intravascular haemolysis is prevented by CD59 and the selective shedding of the membrane attack complex by vesiculation.[32]

Low-level CD59 expression in the present study might be explained by consumption in the face of ongoing complement activation. Malasit and colleagues suggested that bound proteins (IgG, C3 and C5b-9) are not only due to the direct effect of splenectomy but result from activation of the classic pathway by auto-antibodies and the derrangement of regulatory factors due to pathological red cell membrane composition and structure. The latter might result in enhanced susceptibility to spontaneous attack via alternative pathways or bystander mechanisms.[33]

Irrespective of the cause, deposition of potentially cytolytic C5b-9 complexes may be partly responsible for the previously observed increases in membrane permeability to ions that are known to be more prevalent in splenectomised thalassaemia patients and which account for the characteristic intracellular calcium accumulation. When deposition of C5b-9 complexes reaches a critical level, disturbance of ionic homeostasis alters the cells and they are removed in the spleen.[33]

Furthermore, in a study by Kawano,[28] expression of complement regulatory proteins (CD59 and CD55) were found to be markedly decreased on autologous cells undergoing apoptosis, which suggests that these proteins accelerate the clearance of cells by complement-mediated mechanisms.[34] This might explain why CD59 expression across all four groups in the present study was below normal reference values.

Normally, the proportion of CD59-deficient red cells should not be more than 2–4%.[31] As thalassaemic cells are structurally abnormal, apoptosis may occur more frequently. Thus, CD59 expression decreases and they become liable to complement-mediated lysis.[28] However, whether low CD59 expression in thalassaemia patients is due to down-regulation of transcription by the interplay of cytokines or to a relative deficiency of an enhancer protein needs to be verified.

In the present study, CD59 expression showed a negative correlation with reticulocytosis, which is consistent with the

work of Fujioka and Yamada.[35] Also, a negative correlation between CD59 expression and normoblast count denotes ongoing minimal lysis of red cells, which was not apparent clinically. This work suggests that measurement of CD59 expression can be used as a predictor of subclinical complement-activated haemolysis that is insufficient to produce a positive direct antiglobulin test.[9] □

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