The significance of a positive DAT in thalassemia patients

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The DAT is performed for the detection of antibody or complement on the surface of RBCs. Our institution previously performed DATs on all chronically transfused thalassemia patients before each transfusion episode to detect early alloimmunization. The medical records of all thalassemia patients treated at our institution from 2004 to 2007 were reviewed to determine the significance of the high rate of positive DATs (52.5% of 80 patients). The majority of IgG-reactive DATs were associated with a nonreactive eluate (65.4% of 286 eluates performed). A positive DAT was significantly associated with splenectomy ($\chi^2 = 15.4$; $p < 0.001$), elevated IgG levels ($\chi^2 = 26.8$; $p < 0.001$), HCV ($\chi^2 = 20.7$; $p < 0.001$), and warm autoantibody ($\chi^2 = 5.87$; $p = 0.03$). Multivariate analysis revealed that only HCV (OR, 5.0; $p = 0.037$) and elevated IgG levels (OR, 9.0; $p = 0.001$) were independently associated with a positive DAT. Alloimmunized thalassemic patients were more likely to have a positive DAT than nonalloimmunized patients, but this association was not significant (OR, 2.2; $p = 0.11$). A positive DAT did not correlate with decreased response to transfusion, RBC survival, hemolysis, or increased transfusion requirements. Only two cases of early alloimmunization were detected by DAT among 288 DAT-positive samples studied during 4 years. This study demonstrated that the routine performance of DATs on pretransfusion specimens in thalassemic patients has limited clinical utility, and the elimination of this test will improve turnaround time and decrease costs. ***Immunohematology 2010;26:87–91.***

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The DAT detects antibody, most commonly IgG, or complement on the surface of the RBC. Characterization of RBC-bound IgG includes identifying eluate reactivity and specificities. Eluates prepared from the RBCs of patients with autoantibodies (e.g., warm autoimmune hemolytic anemias or autoantibodies induced by certain drugs) can be panagglutinins, reacting with all RBCs. Eluates prepared from the RBCs of patients with serologic or hemolytic transfusion reactions contain IgG alloantibody(ies) with specificity for an RBC antigen. Occasionally, an IgG eluate is not reactive with reagent RBCs. This is thought to be caused by an IgG antibody that is specific for an antigen that is not part of the RBC membrane, an antibody concentration that is too weak to be detected, or an elution method that does not remove the antibody or alters the antibody. It has also been shown that a certain number of IgG molecules normally attach to the erythrocyte surface in a nonimmunologic fashion. The degree of attachment has been observed to have a direct relationship with the concentration of plasma IgG, and at high levels can lead to a positive DAT. This nonimmunologic attachment phenomenon does not tend to cause decreased in vivo survival.[1] Studies have also shown that high serum IgG levels are not always associated with a positive DAT; therefore, factors in addition to an elevated serum IgG level must contribute to a positive DAT and nonreactive eluate.[2] A positive DAT has also been linked with RBC senescence.

Abnormalities in the immune system of children with β-thalassemia major have been previously documented, including an expansion of circulating B cells and a modest polyclonal gammopathy in both splenectomized and nonsplenectomized patients.[3] Immunologic abnormalities in this patient population are thought to be caused by the combined effect of chronic overstimulation of the immune system attributable to allogeneic transfusions, iron overload, and splenectomy.[4] However, high immunoglobulin levels have been found in younger, not yet transfused patients with little iron storage,[5] indicating that the disease itself, and not just treatment-related sequelae, may play a part in the abnormal IgG levels.

Until 2007, a DAT was routinely performed on pretransfusion samples from β-thalassemic patients in our institution with intent to detect early alloimmunization. During a previous quality assurance review, we found only two cases of early alloimmunization detected by this means. Two of 288 positive DATs in thalassemic patients during 4 years reviewed had a new alloantibody in the eluate that was not present in the serum. However, we found that greater than half of our regularly transfused β-thalassemic patients had a positive DAT, a much higher rate than other hospitalized patients. Approximately 1 to 15 percent of hospital patients and 0.01 to 0.1 percent of blood donors have a positive DAT.[6] The cause, clinical significance, and serologic characteristics of the positive DAT by routine laboratory methods in thalassemic patients have not been described. We performed this retrospective review of positive DATs in our thalassemic patients to determine whether a positive DAT in this population is significant for transfusion therapy and to determine whether DAT surveillance is of value in screening chronically transfused subjects for alloimmunization. We hypothesized that the positive DAT is attributable to multiple factors, including elevated IgG levels, and in the majority of cases is unlikely to be associated with immunization otherwise undetectable by routine transfusion testing. Other areas of inquiry included the clinical significance of a nonreactive eluate for future transfusions and the effect on RBC survival.

Materials and Methods

Patient Data

The medical records of 80 β-thalassemia patients chronically transfused between 2004 and 2007 were reviewed. These patients collectively had 5696 transfusions and 288 positive DATs during this period at Weill Cornell Medical Center. Clinical data reviewed included patient age, sex, splenectomy status, IgG level, hepatitis C status, and other clinical history. Antibody screening and identification results, DAT and eluate results (including polyspecific or IgG/C3bC3d), the number of units transfused, and the interval between transfusions were also analyzed. All available laboratory evidence of hemolysis was reviewed, including hemoglobin, hematocrit, haptoglobin, lactate dehydrogenase, and bilirubin levels. Transfusion response was assessed using pretransfusion hemoglobin levels at the subsequent visit. Approval to conduct the study was obtained from the Weill Cornell Medical College Institutional Review Board.

Immunohematology Testing

ABO grouping, D typing, and alloantibody screening were performed by standard semiautomated gel technology (Provue Version 3.14B, Provue/Tecan; Ortho Clinical Diagnostics, Rochester, NY). Alloantibody identification on serum and eluates was performed using standard gel methods (Ortho Clinical Diagnostics and Immucor cell panels; Immucor, Norcross, GA). DATs were performed using IgG heavy chain–specific, rabbit-derived antibody and murine monoclonal C3bC3d. Acid elution was performed using low-pH glycine buffer.

Immunochemistry Testing

Serum IgG concentrations were quantified using nephelometric methods (Beckman Immage; Beckman Coulter, Inc., Brea, CA). The IgG reference range is 1.8 to 3.3 g/dL, and all values greater than 3.3 g/dL were considered elevated.

Screening for hepatitis C status was performed using an enzyme immunoassay (Vitros ECi-Q Immunodiagnostic System; Ortho Clinical Diagnostics). Confirmatory testing was performed using an in vitro qualitative enzyme immunoblot assay (Chiron RIBA HCV Strip Immunoblot Assay; Novartis Vaccines and Diagnostics, Inc., Emeryville, CA). All reactive enzyme immunoassays were confirmed positive by immunoblot.

Statistical Analysis

Categorical variables were assessed by cross tabulation and tested for significance with χ^2 or Fisher's exact test. A probability value of less than or equal to 0.05 was considered significant. Continuous variables were reported as means with standard deviations and were tested for significance with the *t* test. Variables with a two-tailed significance value of $p \leq 0.05$ were included in logistic regression models to explore multivariable significance. Statistical testing was performed using Stata version 10.1 (StataCorp, College Station, TX).

Results

Clinical Patient Characteristics

A total of 80 β-thalassemia patients regularly transfused from 2004 to 2007 were identified. Fifty-nine patients were diagnosed with β-thalassemia major and 21 patients were diagnosed with β-thalassemia intermedia (1 of whom was diagnosed as sickle cell and β-thalassemia intermedia). The majority (53 of 80) of these patients were only treated at our institution for thalassemia for the entirety of the study period. Patient characteristics including diagnosis, age, sex, and race are listed in Table 1. Overall, 68.8 percent (55 of 80) of the patients were splenectomized and 61.3 percent (49 of 80) had an elevated immunoglobulin level (Table 2). All 80 patients were tested for hepatitis C; 36 were positive (45.0%). The mean number of transfusions per patient treated exclusively at this institution during this interval was 80.0 (range, 3–202; Table 3).

Table 1. Patient demographic information and relationship to DAT

Variable	Positive DAT (n=42)	Negative DAT (n=38)	χ^2	p value*
Diagnosis				
β-thalassemia intermedia	14 (33.3%)	7 (10.5%)	2.29	0.13
β-thalassemia major	28 (66.7%)	31 (81.6%)		
Age (years)				
mean	32.9 ± 12.7	20.7 ± 12.1	*t* test	0
range	5–56	2–50	4.39	
Sex				
Female	21 (50%)	24 (63.2%)	1.4	NS
Male	21 (50%)	14 (36.8%)		
Race				
Asian	6 (14.3%)	16 (42.1%)	OR	0.006
Caucasian	36 (85.7%)	22 (57.9%)	4.4	

*χ^2 or Fisher's exact test used unless otherwise specified.

Table 2. Chi-square analysis of clinical variables associated with a positive DAT

Variable	Positive DAT n (%)	Negative DAT n (%)	χ^2	p value
Splenectomy status				
Spleen absent	37 (88.1)	18 (47.4)	15.4	<0.001
Spleen present	5 (11.9)	20 (52.6)		
HCV status				
HCV positive	29 (69.0)	7 (15.8)	20.7	<0.001
HCV negative	13 (31.0)	31 (81.6)		
Gamma globulin				
Elevated IgG level	37 (88.1)	12 (31.6)	26.8	<0.001
IgG within reference range	5 (11.9)	26 (68.4)		

Table 3. Chi-square analysis of transfusion and laboratory variables associated with a positive DAT

Variable	Positive DAT	Negative DAT	χ^2	p value*
Alloimmunization				
Alloimmunized	17 (40.5%)	9 (23.7%)	2.56	0.11
Not immunized	25 (59.5%)	29 (76.3%)		
Warm autoantibody				
Present	6 (14.3%)	0 (0%)	5.87	0.027
Absent	36 (85.7%)	38 (100%)		
Total number of transfusions/patient†				
mean	86.0 ± 48.4	72.1 ± 51.9	*t* test	NS
range	8–202	3–168	1.24	

*χ^2 or Fisher's exact test used unless otherwise specified.
†Data includes only patients exclusively transfused at our institution.

Laboratory Patient Characteristics

In addition to type and screen, a DAT (and eluate as required) was performed before each transfusion according to our institution's standard operating procedure. Forty-two patients had a positive DAT on at least one occasion, and 38 patients had no history of a positive DAT during the study period. The majority, 96.2 percent, of the positive DATs were positive owing to IgG (277 of 288) only, 0.7 percent to complement (2 of 288) only, and 3.1 percent to both IgG and complement (9 of 288). Of the 286 eluates performed, 65.4 percent (187 of 286) were associated with a nonreactive eluate, 29.7 percent (85 of 286) with a panagglutinin, 2.1 percent (6 of 286) with nonspecific reactivity, and 2.8 percent (8 of 286) with an alloantibody. The 85 DATs with a panagglutinin were from the same 6 patients with a warm autoantibody.

Alloimmunization and Autoimmunization Rates

The overall rate of alloimmunization was 32.5 percent (26 of 80) in this patient population. More than one alloantibody was detected in 69.2 percent (18 of 26) of immunized patients. Table 4 presents the specificities of clinically significant alloantibodies by blood group antigens. Only 2 cases of early alloimmunization to E and Jk^a were detected by a positive DAT only (negative serum antibody screen). Warm autoantibodies were detected in 7.5 percent (6 of 80) of all patients. Only 5 percent (4 of 80) of all patients were found to have both an alloantibody and an autoantibody.

Clinical and Demographic Factors Associated With a Positive DAT

The DAT-positive and DAT-negative groups were similar with respect to sex. The DAT-positive patients were significantly older than the DAT-negative patients (32.9 ± 12.7 versus 20.7 ± 12.1 years; *t* test = 4.39; p = 0). There was a significant association between race, Caucasian versus Asian, and positive DAT (OR, 4.4; 95% CI, 1.52–12.46; p = 0.006). However, this difference was determined to be attributable to HCV status and age by logistic regression modeling. Although thalassemia intermedia patients were more likely to have a positive DAT (OR, 2.2) this was not statistically significant (p = 0.13).

Table 4. Alloantibody specificities of DAT-positive and DAT-negative patients

Antibody specificity	No. of patients with alloantibodies		
	DAT-positive	DAT-negative	Total
D	0	1	1
E	8	5	13
e	1	0	1
C	1	2	3
c	1	2	3
K	10	3	13
Jk^a	1	2	3
Jk^b	1	0	1
Fy^a	1	0	1
Fy^b	1	0	1
M	1	0	1
Low incidence			
Bg^a	3	0	3
Kp^a	2	1	3
C^w	1	0	1
V	3	0	3
VS	1	0	1
Js^a	2	0	2
Co^b	1	0	1
DAK	1	0	1

A positive DAT was associated with splenectomy (p < 0.001), elevated IgG levels (p < 0.001), HCV (p < 0.001), and warm autoantibody (p = 0.03) in univariate testing (Tables 2 and 3). When adjusted for the effect of age, race, immunoglobulin level, and splenectomy status in logistic regression stepwise modeling, HCV was significantly associated with a positive DAT (OR, 5.0; p = 0.037). When adjusted for the effect of age, race, HCV, and splenectomy status in logistic regression stepwise modeling, an elevated IgG level was also significantly associated with a positive DAT (OR, 9.0; p = 0.001).

The transfusion response was similar in the DAT-positive and DAT-negative groups as determined by review of pretransfusion values at each visit. There was no clinical or laboratory evidence of hemolysis in any of the patients with a positive DAT (data not shown). Similarly, the total number of transfusions received at our institution between the two groups was not significantly different, suggesting there was no increased transfusion requirement among DAT-positive patients as a result of either hemolysis or other clinical factors (Table 3).

Laboratory Factors Associated With a Positive DAT

Seventeen of the 42 patients (40.5%) with a history of a positive DAT were alloimmunized, and 9 of the 38 patients (23.7%) with no history of a positive DAT were alloimmunized.

Alloimmunized thalassemic patients were more likely to have a positive DAT than nonalloimmunized patients (OR, 2.2); however, this was not statistically significant (p = 0.11). All of the patients with a warm autoantibody had a positive DAT (p = 0.03). Blood group B patients were 2.6 times less likely to have a positive DAT compared with other blood groups (χ^2, 3.28; p = 0.07). (Table 5)

Table 5. Frequency of ABO groups

DAT group	A	B*	AB	O
DAT+	13	7	2	20
DAT–	9	13	1	15

*χ^2 = 3.28; p = 0.07.

Discussion

The DAT may be used as a sensitive method to detect a hemolytic transfusion reaction, hemolytic disease of the fetus and newborn, or early alloimmunization, or to diagnose autoimmune hemolytic anemia. In this patient population, the high rate of positive DATs could not be explained by any of these alloimmune phenomena, because the majority (65.4%) of IgG-reactive DATs had a nonreactive eluate. Clinical and laboratory factors were analyzed to see whether a relationship existed between a positive DAT and age, race, transfusion frequency, blood group, hepatitis C, splenectomy status, IgG level, blood group, and alloimmunization or autoimmunization status. The following variables were significantly associated with a positive DAT: older age, Caucasian race, elevated gamma globulin levels, presence of anti-HCV antibody, splenectomy, and presence of a warm autoantibody. Because HCV and a splenectomized state are known to cause elevated IgG levels and older patients were more likely to be HCV positive, a multivariate analysis was performed to determine whether age, race, HCV, splenectomy, and IgG level were independent risk factors for a positive DAT. This analysis revealed that the only true independent risk factors were an increase in IgG levels and HCV positivity. The association between increased age and positive DAT may be attributable to the increased likelihood of HCV positivity in these patients as many were multiply transfused before the institution of HCV serologic testing of blood donors in 1992. The possible association with a positive DAT and Caucasian race appears to be related to the fact that the non-Caucasian patients were younger (median, 16 years of age versus median, 35 years of age) and therefore less likely to be HCV positive. Patients with blood group B were less likely to have a positive DAT, and patients alloimmunized to RBC antigens were more likely to have a positive DAT, but these associations were not statistically significant.

Laboratory and clinical data were reviewed to determine whether there was any clinical significance of a positive DAT with a nonreactive eluate. However, transfusion increments were normal, and no evidence of hemolysis or transfusion reaction was noted in the DAT-positive patients. Only 2 of 288 positive DATs during the period studied revealed a new alloantibody that was not detected in serum.

It was noted during our study that the rate of alloimmunization in our β-thalassemia patients (32.5%) was at the higher end of the spectrum of those previously reported; alloimmunization rates described previously range from 3 to 37 percent.[7–10] This variability can be explained by differences in RBC antigen matching methods across previous studies. Some institutions match for DCcEeK in their sickle cell and thalassemia patients. We currently match only for ABO and D. Furthermore, we use a more sensitive method of antibody detection than that used in many of the older studies (gel versus tube methodology).[7–10]

It is known that β-thalassemia patients have high levels of serum IgG, even in patients not yet transfused. Splenectomized thalassemia patients have even higher levels of IgG.[5] Multiple studies in general patient populations have shown that increased serum IgG levels are associated with a positive DAT and a nonreactive eluate, as seen in our study in thalassemic patients.[1,2,11,12] In a very small study, Garratty[6] previously reported increased IgG bound to RBCs in thalassemic patients. He found increased numbers of IgG molecules bound to RBCs by the complement fixation antiglobulin consumption assay in 6 of 9 patients with thalassemia syndromes. There is debate, however, as to whether this bound antibody is caused by cytophilic IgG or autoantibody.

The aging of a circulating RBC is associated with a 20 to 30 percent reduction in sialic acid within the RBC membrane. It has been suggested that the cells of the reticuloendothelial system recognize a determinant on the aging RBCs produced after sialic acid removal.[6] Evidence also suggests that RBC-bound IgG may be responsible for the removal of senescent human RBCs. Thus, it has been theorized that loss of sialic acid creates a "senescent cell antigen" that binds to an autologous IgG antibody specific for that antigen.[13] Once IgG is bound, the senescent cells are quickly phagocytosed by macrophages and removed from circulation.[6] The site and molecular changes of the senescent antigen have not been definitively described.

Kahane et al.[14] found that sialic acid residues on the RBC surface RBC of thalassemic RBCs are distributed in an uneven manner and are less abundant than those present on normal RBC surfaces. It follows that thalassemic RBCs would also have an exposure similar to that of the senescent cell antigen and IgG could bind to these sites on thalassemic RBCs. In this case the antigen would not be caused by senescence, but perhaps be attributable to defective membrane biogenesis or an enhanced rate of removal of sialic acid from the thalassemic membrane. An autologous antibody may also bind to these sialic acid–depleted membranes, signaling to macrophages that such cells should be removed from circulation.[15] Similarly, low levels of sialic acid in RBC membranes have been reported in patients with sickle cell anemia.

Therefore, it is not surprising that we would find such a high prevalence of positive DATs with IgG bound and

nonreactive eluates in this patient population. The question that still remains to be answered is the following: if all thalassemic RBCs have reduced sialic acid, why do only half of our patients with thalassemia have antibody bound to their RBCs? The answer to this question may be found in the complexity and diversity of the immune system, as well as in the clinical and molecular heterogeneity of thalassemia. Epigenetic and genetic factors most likely play a role in RBC autoimmunization as they do in the regulation of RBC alloimmunization. In addition, the frequency with which a patient is transfused and the percentage of autologous RBCs in a patient's circulation may affect the occurrence of a positive DAT. From data gathered in our study, however, this last explanation cannot be the sole reason for a positive DAT, as the most frequently transfused patients were not more likely to have a positive DAT. It does seem that either underlying genetic or epigenetic factors have a role in the positive DAT autoimmunization phenomena, as the occurrence of a positive DAT differed by race and blood group.

It was not unexpected that elevated levels of IgG in thalassemia patients would lead to a positive DAT with a nonspecific eluate. However, it was unexpected that patients with hepatitis C would have a positive DAT independent of their IgG level. This is most likely related to the autoreactive manifestations associated with HCV infection, such as autoantibody production, cryoglobulinemia, and thyroid disorders. It may be that autoantibodies or antibodies cross-reactive with common RBC antigens are transiently present and account for the temporary positive DATs in these patients. This requires further investigation.

Performing routine DATs on pretransfusion specimens in thalassemic patients has limited clinical utility. The positive DAT reflects the pathophysiology of this disease in the majority of patients and does not predict decreased RBC survival. Although eliminating this testing may miss a new, low-titer alloantibody, this occurrence would be very rare, and the antibody probably clinically insignificant, as current antibody screening methods are quite sensitive. Eliminating the pretransfusion DAT in this patient population will improve turnaround time and decrease costs for the patient and health-care system.

References

1. Huh YO, Liu FJ, Rogge K, Chakrabarty L, Lichtiger B. Positive direct antiglobulin test and high serum immunoglobulin G values. Am J Clin Pathol 1988;90:197–200.
2. Toy PTCY, Chin CA, Reid ME, Burns MA. Factors associated with positive direct antiglobulin tests in pretransfusion patients: a case-control study. Vox Sang 1985;49:215–20.
3. Dwyer J, Wood C, McNamara J, et al. Abnormalities in the immune system of children with beta-thalassaemia major. Clin Exp Immunol 1987;68:621–9.
4. Kapadia A, de Sousa M, Markenson AL, Miller DR, Good RA, Gupta S. Lymphoid cell sets and serum immunoglobulins in patients with thalassaemia intermedia: relationship to serum iron and splenectomy. Br J Haematol 1980;45:405–16.
5. Tovo PA, Miniero R, Barbera C, Sacchetti L, Saitta M. Serum immunoglobulins in homozygous β-thalassemia. Acta Haematol 1981;65:21–5.
6. Garratty G. The significance of IgG on the red cell surface. Transfus Med Rev 1987;1:47–57.
7. Coles SM, Klein HG, Holland PV. Alloimmunization in two multitransfused patient populations. Transfusion 1981;21:462–6.
8. Rebulla P, Modell B. Transfusion requirements and effects in patients with thalassaemia major. Cooleycare Programme. Lancet 1991;337:277–80.
9. Singer ST, Wu V, Mignacca R, Kuypers FA, Morel P, Vichinsky EP. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly Asian descent. Blood 2000;96:3369–73.
10. Wang LY, Liang DC, Liu HC, et al. Alloimmunization among patients with transfusion-dependent thalassemia in Taiwan. Transfus Med 2006;16:200–3.
11. Heddle NM, Kelton JG, Turchyn KL, Ali MAM. Hypergammaglobulinemia can be associated with a positive direct antiglobulin test, a nonreactive eluate, and no evidence of hemolysis. Transfusion 1988;28:29–33.
12. Szymanski IO, Odgren PR, Fortier NL, Snyder LM. Red blood cell associated IgG in normal and pathologic states. Blood 1980;55:48–54.
13. Galili U, Flechner I, Rachmilewitz EA. A naturally occurring anti-alpha-galactosyl IgG recognizing senescent human red cells. Prog Clin Biol Res 1985;195:263–78.
14. Kahane I, Polliack A, Rachmilewitz EA, Bayer EA, Skutelsky E. Distribution of sialic acids on the red blood cell membrane in beta thalassaemia. Nature 1978;271:674–5.
15. Galili U, Korkesh A, Kahane I, Rachmilewitz EA. Demonstration of a natural antigalactosyl IgG antibody on thalassemic red blood cells. Blood 1983;61:1258–64.

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