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The spectrum of underlying diseases in patients with positive-Direct Antiglobulin Test

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Abstract

Background: The association between a positive direct antiglobulin test (DAT) and malignancy has been recognized. However, there has been dissimilar data on the spectrum of underlying malignancies and the timing of occurrence.

Objective: To estimate the pattern of underlying diseases over a 12 year-period.

Methods: All consecutive patients with positive-DAT were selected and a follow-up for concomitant or subsequent development of malignancy, infection and autoimmune disease was conducted. The gel technique with 5 monospecific Anti-IgG, anti-IgA, anti-IgM, anti-C3c and anti-C3d was used.

Results: Between 2009 and 2021, 164 patients, 86 males and 78 females, were found to have a positive DAT among 12200 patients subjected to testing. The mean age was 62 years. Seventy two patients had autoimmune hemolytic anemia (AIHA). The spectrum of underlying diseases included: Lymphoproliferative disorders (14%), myeloproliferative disorders (10.3%), solid tumors (18.3%), autoimmune diseases (11%) and infectious disease. The most frequently observed condition was myelodysplastic syndrome at all stages.

The strength of DAT reaction was correlated with the degree of hemolysis but not with the stage of malignancy.

Conclusion: A positive-DAT suggests the presence of underlying malignancy, mainly hematological and even in early stages. All patients with MDS should be tested for DAT in diagnostic and therapeutic intent.

Introduction

The Direct antiglobulin test (DAT) is used to detect the presence of red blood cell- coated antibody and/or complement. Routine DAT is performed using polyspecific antiglobulin reagent containing anti-IgG, IgA, IgM, C3d and C3b. Once a positive-DAT is found, monospecific reagents are used to determine the specific type of antibody.

In the presence of clinical and biological stigmata of hemolysis, DAT may help in diagnosing autoimmune hemolytic anemia (AIHA). A positive-DAT without evidence of hemolysis has been reported in 0.1% to 1.4% of blood donors and 1% to 3.5% of unselected hospitalized patients [1-5]. AIHA may be primary or associated with an underlying disease. It is a rare event occurring in 1/35000 to 1/80000 individuals yearly [6,7] at any age and has female predominance. The idiopathic form occurs mostly between 15 and 40 years whereas the secondary form occurs later in life. According to the first largest published studies 41% to 65% of AIHA were considered to be idiopathic, 22% to 40% secondary to underlying diseases and 15% to 19% drug-induced [7,8].

The most common types of autoantibodies involved in AIHA are warm antibodies (75%-90%) that react optimally with human RBCs at 37^{0}C. In warm AIHA, the DAT is positive with IgG in 20% to 66%, with IgG and complement component C3d in 24% to 64% and with C3d alone in 7% to 14% of cases [9-12]. There is a recognized bi-directional relationship between autoimmune disease (AID) and lymphoproliferative disorders; especially non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) [13-17]. Myeloproliferative neoplasms (MPN) and myelofibrosis have also long been associated with AIHA [18-20]. A positive DAT in patients with multiple myeloma (MM) was most frequently observed with IgG, and usually produced little hemolysis [21].

The increased incidence of a positive DAT in myelodysplastic syndrome (MDS), up to 50%, is thought to be a manifestation of disturbed immune homeostasis and might support a more rational use of steroid therapy in these patients [22-30].

Positive-DAT was found in 11% of AML patients at the time of diagnosis regardless of FAB morphological subtype and may develop subsequently during remission or progression [31].

It is known that solid malignant tumors can trigger the development of auto-antibodies against proteins in different organ systems that cause paraneoplastic syndromes. In many AID autoantibodies may develop against multiple organs including the blood system. The availability of DAT has rendered the diagnosis of AIHA easier, however the mechanism by which this self-tolerance is deregulated remains still not fully understood.

An underlying disease has been found in 51% to 77% of positive DAT-cases with or without AIHA [32-37]. Limited data has been reported on the timing between the diagnosis of positive-DAT and the occurrence of underlying malignancy. Genty, *et al.* have studied the

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Table 1. Demographic, clinical and laboratory characteristics of DAT-positive patients

Clinical and biological characteristics	
Mean age (ranges)	62 years [19 – 86 years]
Sex ; ratio	77 females; 86 males
Underlying disease	124 (76%)
No underlying disease	39 (24%)
History of splenectomy	4 (2.5%)
Reported splenomegaly	26 (14.5%)
Associated thrombocytopenia	55 (34.5%)
Associated neutropenia	41 (25.5%)
IgG	130 (80%)
C3d	10 (6%)
C3d & IgG	19 (11%)
IgA	2 (1.5%)
Positive DAT & positive IAT	31 (19%)

bone marrow of patients with AIHA and were able to show that AIHA may precede the onset of NHL by months or years [32].The strength of the DAT does not always predict the biological activity of antibodies. However, substantial studies tend to support a direct correlation between the strength of the DAT result and hemolysis [33].

In order to define the spectrum of underlying malignancies and analyze the chronology of their occurrence with the occurrence of positive-DAT, we have conducted a retrospective study of all consecutive patients with positive DAT due to warm autoantibodies diagnosed and treated in our institution over an 11-year period.

Patients and methods

Patient selection

All consecutive patients above 19 years of age with positive-DAT between 2009 and 2020 were selected for this study after an institutional review board approval. The DAT was performed upon physician request if hemolysis was suspected or in case of positive auto control in antibodies identification. The diagnosis of AIHA was made if the hemoglobin level was below 12 g/dl in men and 11 g/dl in women, the haptoglobin level was below 0.34 g/l and spherocytosis were present on blood smear.

Epidemiological, clinical and biological data for each patient were collected and recorded in a study specific case report form. This group of patients constituted a cohort and subjected to a long term follow up.

Underlying malignancies were especially looked for. Pathology report and bone marrow studies were reviewed. Collagen disorder and systemic lupus erythematous (SLE) were diagnosed using specific serology tests. All positive warm DAT cases were included regardless of drug intake.

Positive-DAT may be due to the presence of alloantibodies in recently transfused patients [12]. No patient had received blood product transfusion within the last 15 days before the IAT and DAT were carried out.

Methods and statistical analysis

In routine pre-transfusion testing in our institution, ABO grouping, Rh typing, antibody screening and a cross match are performed. If the antibody screening is positive, then antihuman globulin (AHG) crossmatch is performed using selected donor units known to be negative for the corresponding antigens. In patients with pan-reactive panel and a positive auto-control, DAT is automatically performed.

The gel technique using BIO-RAD ID-Card "DC-Screening I" consisting of five different mono-specific AHG reagents; anti-IgG, anti-IgA, anti-IgM, anti-C3c (all rabbit), and anti-C3d (Monoclonal cell line C139-9) suspended in gel, and the negative control was used for DAT. Antibody screening was done with commercial 3 Cell panel (ID Dia cell-I-II-III Asia) Gel card. The complete phenotype was determined either at diagnosis or subsequently within 3 weeks after corticosteroid treatment (D, C, E, c, e, C^w, K, k, Kp^a, Kp^b, Js^a, Js^b, Jk^a, Jk^b, Fy^a, Fy^b, Le^a, Le^b, P1, M, N, S, s, Lu^a, Lu^b and Xg^a); WBC-reduced donor RBCs matched with the patient's phenotype were provided for transfusion.

To determine whether positive-DAT occurred more frequently in patients with a specific disease or whether these were chance associations, contingency tables were drawn up for every underlying disease encountered in this series. The data were subjected to statistical analysis using Chi squared (χ^2) test with Yates' correction at one degree of freedom; the significance level was set at $p < 0.05$ (Table 1).

Results

12200 patients were subjected to testing, 164 patients (1.34%) were found to have a positive-DAT with warm autoantibodies. Seventy two patients (43.8%) had AIHA, 28 of them (40%) had hemoglobin level below 7.5 g/dl. The median age was 62 years (range: 19 to 86 years). There were 77 females and 86 males. The median follow-up time was 90 months (18 to 132 months).

The majority of patients with AIHA (83.5%) had underlying disease versus 68% of those without AIHA. Twenty nine patients (15.5% of positive-DAT patients) had no AIHA and no underlying disease whereas only 12 patients (7% of positive-DAT patients) had AIHA but no apparent underlying condition.

Hematologic disorders of both lymphoid and myeloid origin, autoimmune diseases and solid tumors were respectively found in 24.4%, 18.3% and 11% of positive-DAT patients (figure 1). Although, many patients had concomitantly two different conditions the most frequently observed hematological condition was MDS (17 cases),

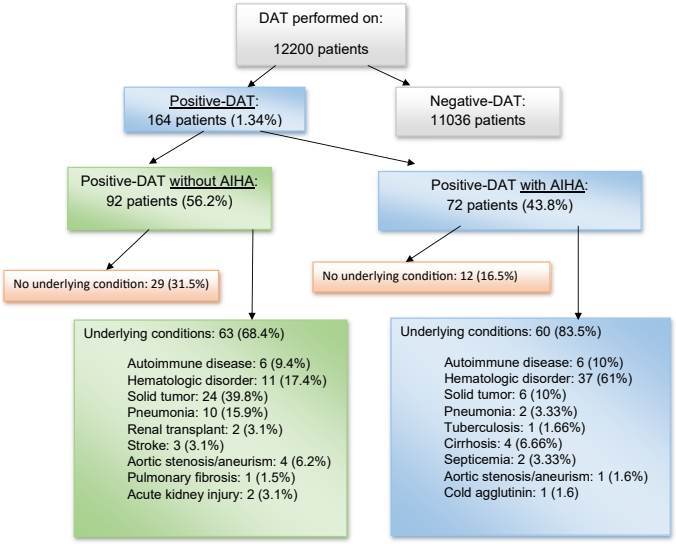

Figure 1. Diagram illustrating the positive DAT population, the prevalence of AIHA and the presence of underlying diseases. (Percentages of patients in the whole cohort)

followed by MM (8 cases), NHL (6 cases), CLL (5 cases), MPN (5 cases) and AML (4 cases). Table 2 shows the numbers of patients with the different underlying diseases compared to the total numbers of patients with the same condition treated in the institution during the same period. Among the underlying solid tumors, only the renal cell carcinoma was significantly associated with the development of positive DAT.

The strength of DAT reaction was correlated with the presence of hemolysis in the entire cohort. A strong reaction (+++/++++) was seen in 75% of MM, 50% of NHL and CLL and 33% of MDS patients. The DAT was also strong in patients with autoimmune disease particularly autoimmune hepatitis (2 patients), SLE (2/6 patients) and one patient with autoimmune myelofibrosis associated with diffuse large B-cell lymphoma (Table 3).

Table 2. Hematologic malignancy and the chronology of positive-DAT occurrence

Chronology of DAT occurrence							
Hematologic Disorders	Diagnosis		AIHA	No AIHA	Total Positive-DAT	Positive-DAT at presentation	Subsequent Positive-DAT
Myeloid Disorders	MDS		16	1	17	13 (76%)	4 (23%)
		MDS	10	1	11	8 (47%)	3 (17.5%)
		MDS+MPN	2	0	2	1 (5.5%)	1 (5.5%)
		MDS+AML	3	0	3	3 (18%)	0
		MDS+CML	1	0	1	1 (5.5%)	0
	AML		3	0	3	3 (100%)	0
	MPN		3	2	5	3 (60%)	2 (40%)
Lymphoid Disorders	MM		4	4	8	1 (12.5)	7 (87.5%)
	NHL		3	3	6	5 (83%)	1 (17%)
	HD		2	0	2	2 (100%)	0
	ALL		2	0	2	2 (100%)	0
	CLL		4	1	5	2 (40%)	3 (60%)

Table 3. Underlying disease categories in positive-DAT patients and the significance of x^2 test with "Yates" correction for each category calculated according to the total number of DAT performed (12200), the total number of cases registred in the institution over the same period and the total number of positive-DAT

Disease Category	Number (%)	Underlying disease	**Positive DAT**	**AIHA**	**No AIHA**	**Number of inpatients (same period)**	**%**
B-cell & plasma cell malignancies	23 (14%)	.					
		Non-Hodgkin's Lymphoma	**6**	**3**	**3**	**195**	**3%**
		Hodgkin's disease	**2**	**2**	**0**	**49**	**4%**
		Acute Lymphoblastic Leukemia	**2**	**2**	**0**	**12**	**15%**
		Chronic Lymphocytic Leukemia	5	4	1	300+	**ND**
		Multiple myeloma	8	4	4	79	10%
Myeloid disorders	27 (10.3%)						
		Myelodysplastic Syndrome	**17**	**16**	**1**	**207**	**8%**
		Acute Myelogenous Leukemia	4	**4**	**0**	**70**	**5.6%**
		Chronic Myelogenous Leukemia	**1**		**1**	**13+**	**ND**
		Myeloproliferative Neoplasms	5	3	2	57+	ND
Malignant solid tumors	30 (18.3%)						
		Breast cancer	**9**	**0**	**3**	**600+**	**ND**
		Prostate cancer	**3**	**0**	**3**	**250+**	**ND**
		Pancreatic cancer	1	0	1	99+	**ND**
		Renal Cell Carcinoma	**2**			**45**	**4.5%**
		Lung cancer	**1**	**0**	**1**	**290+**	**ND**
		Gastric cancer	1	0	1	60+	**ND**
		Bladder cancer	**1**	**0**	**1**	**100+**	**ND**
		Colon cancer	**3**	**0**	**3**	**300+**	**ND**
		Ovarian cancer	1	0	1	**60+**	**ND**
		Liver cancer	**4**	**0**	**4**	**60+**	**ND**
		Esophageal cancer	**3**	**1**	**2**	**30+**	**ND**
		Soft Tissue Sarcoma	1	0	1	30+	ND
Autoimmune diseases	18 (11%)						
		Systemic Lupus Erythematous	**6**			**29**	**20.5%**
		Auto-Immune Hepatitis	**3**			**12**	**25%**
		Small Vessel Vasculitis	**2**				ND
		Auto-Immune Myelofibrosis	**1**				ND
		Rheumatoid arthritis	**5**			**50**	**10%**
		Auto-Immune Thyroiditis	**1**			8	ND
Renal transplants	2 (1.5%)		2	0	2	25	**8%**

Regarding the patterns of reactivity (Table 4), the most common pattern of reactivity was with anti IgG. The strength of reaction was correlated with the hemolysis. Most patients without evidence of hemolysis had a + to ++ strength of DAT reaction. Specific treatment of the underlying disease with chemotherapy has significantly improved the severity of AIHA and lead to complete reversal of DAT in 70% of cases.

Clinical and biological characteristics of patients with positive DAT

Characteristics of patients are summarized in table 1. Splenomegaly was reported in 26 patients (14.5%). Four patients (2.5%) had had a splenectomy; one for congenital dyserythropoietic anemia, 2 for autoimmune cytopenia and one for traumatic splenic injury. Thrombocytopenia was present in 55 patients (34.5%) and was part of the autoimmune process (Evan's syndrome) in 6 cases.

One hundred and twenty three cases (75%) were considered to be secondary to an underlying disease as shown in figure 1. Forty-one cases (25%) were considered as primary. Most of these patients had been admitted for surgical purpose. Among them thirteen patients (31%) were taking no medication and had no chronic disease (mean age 50 years, range: 19 to 72 years) and 28 patients (69%) had diabetes, coronary artery disease and renal failure (mean age 77 years, range: 50 to 81years). Forty percent of patients had no history of previous transfusion.

A hematologic non lymphoid disorder was found in 27 patients (10.3%) including myeloproliferative neoplasm, myelodysplastic syndrome and acute myeloblastic leukemia. B-cell and plasma cell malignancy were found in 23 patients (14%). Active solid tumor under specific treatment was found in 30 patients (18.3%).

Positive-DAT was present in 76% of MDS patients at presentation while 87% of MM patients developed positive-DAT subsequently during treatment and follow-up.

Our results showed a significant association between the occurrence of positive-DAT and MM, MDS and AID. However no significant association was found with solid malignant tumors apart from renal cell carcinoma.

The types of autoantibodies identified were as follows: IgG: 80%, C3d: 10%, both IgG and C3d: 19% and IgA (1.5%).

Patients with underlying lymphoma were more likely to be associated with erythrocyte coated- IgG and- C3d autoantibodies when compared to multiple myeloma patients who were more likely to develop IgG only erythrocyte autoantibody. C3d only coated RBCs was found in patients with SLE or without underlying disease.

Thirty-one patients (18.5) with positive DAT were IAT reactive. No patient had received blood product transfusion within the last 15 days before the IAT and DAT were carried out. A complete phenotype was determined in all 31 cases (Table allo-antibodies) and these patients received prophylactic antigen matched-RBCs and white blood cell-reduced transfusion (mean: 4 units/patients). Patients with AID and NHL were more likely to develop alloantibodies than patients with MM or MPN. Although, all the 4 MDS patients who developed positive-DAT subsequently had a history of multiple transfusions, only 2 of them (50%) had alloantibodies identified. Table 5 summarizes the alloantibodies detected at the first attempt or after 2 to 3 weeks of corticosteroid treatment.

No patients developed malignancy subsequently to the diagnosis of positive-DAT however; the subsequent occurrence of a positive-DAT was an indicator of disease progression and refractoriness to treatment, especially in MDS patients.

Discussion

It is known that AIDs are associated with or preceding the development of lymphoproliferative diseases .The DAT might be positive at some time during the course of CLL in up to 35% of cases [14] and could be an adverse prognostic factor in stage A [15].

The prevalence of CLL for the same period in our institution was difficult to evaluate because of the ambulatory basis of the follow-up, thus the positive-DAT proportion was difficult to estimate. However; 3% of NHL patients, 15% of ALL patients and 10% of MM patients had positive-DAT. Seven of the 8 MM patients had clinical and biological evidence of hemolysis regardless of the bone marrow infiltration by the plasma cells. AIHA was considered as a rare manifestation in patients with MM [21] furthermore DAT may be falsely positive in IgG multiple myeloma due to the cross-reactivity with the monoclonal Ig or in patients treated with anti-CD38.

Case series identified wide spectrum of autoimmune disorders, seemingly unrelated, in patients with MDS [22-30]. A positive-DAT

Table 4. Strength of reactivity correlated with the erythrocyte auto-antibody type and the presence of severe hemolytic anemia (hemoglobin level ≤ 7.5 g/dl)

DAT strength/Severity of hemolysis/ autoantibody							
		IgG		**IgG + C3d**		C3d	
	Total Nb	**Nb**	**Severe hemolysis**	**Nb**	**Severe hemolysis**	**Nb**	Severe hemolysis
+	**65 (40%)**	51	11 (21%)	11	6 (55%)	3	0
++	**65 (40%)**	60	24 (40%)	3	1 (33%)	2	0
+++	**24 (14.5%)**	20	13 (65%)	2	1 (50%)	2	1 (50%)
++++	9 (5.5%)	4	4 (100%)	4	4 (100%)	1	1 (100%)

Table 5. Alloantibodies identification in allo-immunized patients with positive-IAT and positive-DAT

Allo-antibody specificity in allo-immunized patients:			
Anti-C	3	9.7%	
Anti-D	5	16%	
Anti-E	5	16%	
Anti-Fya	1	3%	
Anti-Jka	1	3%	
Anti-Kell	11	36%	
Anti-M	5	16%	All biphasic and clinically significant

was found in approximately 8% of MDS patients with IgG and or C3 on the RBCs [28] which is in concordance with our results. Patients with early MDS; RA and RARS show a higher incidence of anti-erythrocyte allo- and auto- antibodies and manifest overt non-organ specific autoimmune disorders. Furthermore, MDS share some of the features of aplastic anemia, a disease with an established autoimmune pathogenesis [29,30] and 10% to 20% of patients with MDS present with autoimmune disease (AID), which can be challenging to recognize.

A positive-DAT was found in 19% of agnogenic myeloid metaplasia cases but also, less frequently in Polycythemia Vera. In AML the incidence of positive-DAT was found to be about 11% with only C3 on RBCs and anti-I elute specificity [31]. Our series includes 4 AML cases with no auto- or alloantibody specificity.

Post-transplant immune-mediated hemolysis with positive-DAT is a known phenomenon that may be due to the immunosuppressive therapy that leads to an unbalanced B and T cell lymphopoiesis and the development of RBC autoantibodies.

Little has been reported about AIHA preceding the development of solid tumor [38-40]. Subsequent development of malignancy in patients with AIHA has rarely been addressed outside of scattered case studies [40]. An increased risk of malignancy has been reported among donors with positive-DAT [40]. Cases of paraneoplastic AIHA in solid tumor have been reported. Puthenparambil, *et al.* [38] reviewed 52 of them and found that AIHA may occur prior to, concurrent with and well after the treatment of cancer, 70% of the patients had warm autoantibodies and 30% cold antibodies, some patients had multi-lineage (Evans syndrome) or even multiorgan autoantibodies, and it may occur in every type of cancer but more commonly in renal cell carcinoma which is in agreement with our results. Paraneoplastic manifestations may lead to an early detection of cancer and therefore be a favorable prognostic factor but also it may indicate a recurrence or an advanced stage.

Non-malignant conditions found to be significantly associated with positive-DAT: Pneumonia, Aortic stenosis and or aneurism, Liver cirrhosis and heavy alcoholism, TB, pulmonary fibrosis and EBV infection in acute phase with high serum IgM.

Patients with a positive-DAT differ widely with respect to clinical manifestation; they may have no sign of hemolysis or may present severe hemolysis. A positive-DAT does not distinguish conclusively autoantibodies of clinical importance from those without

IgA autoantibodies were detected in two of our patients (1.5%), IgG was detected in 130 patients (80%%), IgG and C3d in 19 patients (11%) and C3d in 10 patients (6%). These results are in agreement with published data [25,35] however IgA are reported to be present in 14% of warm-type AIHA, almost always associated with IgG and/or IgM antibodies [30].

In our study, the DAT reactivity was stronger in patients with AID than those with hematologic malignancy in whom the degree of anemia was also dependent on the bone marrow ability to produce RBCs. The strength of DAT reaction was also correlated with the severity of hemolysis (Table 4). This correlation has been reported in some studies [34, 35] but was not found in others [33].

Thrombocytopenia was found in 34.5% of our cases, most of them were because of bone marrow failure (MDS, myelofibrosis), and 6 cases were considered as secondary Evans syndrome. The mechanism by which autoimmune thrombocytopenia develops may be similar to AIHA. There is no "gold standard" test that can reliably establish the diagnosis of ITP. Response to corticosteroid therapy or intravenous immunoglobulin is supportive of the diagnosis but a positive response does not exclude secondary ITP. Evans syndrome may show or precede a variety of underlying diseases which may influence both the management and the outcome [36]. Positive-DAT has been found in 22% of ITP cases [39] even without apparent anemia.

Studies on monospecific antiglobulin reactions have failed to attribute a particular underlying disease to a specific autoantibody [39]. The presence of IgG only is more commonly seen in drug-induced autoimmune hemolysis, complement only may reflect a more severe condition and both may be associated with the occult form or the apparent severe hemolysis. No disease specificity for auto- or alloantibody was found in our study.

In our study, alloantibodies were identified in 31 cases (19%). Alloantibodies against RBCs have been shown to be present in a large proportion (25%-47%) of sera from patients with AIHA [33,41]. A complete phenotype for patients with warm autoantibodies is very helpful for the blood bank to provide prophylactic antigen-matched donor RBCs for these patients who are at high risk for alloimmunization and delayed hemolytic transfusion reactions. All 5 cases of anti-M antibody identified were biphasic and considered as clinically significant. All these patients received "M"-antigen negative blood transfusion and no delayed hemolytic reaction was observed.

Our study sheds some light on the significance of AIHA and its relationships with underlying inflammatory or neoplastic conditions. A more precise definition of the spectrum of underlying diseases may lead to a better understanding of DAT positivity and to elaborate a surveillance plan or a screening program for malignancy in these patients.

Despite the retrospective design of the study and the small size of this cohort that allow few statistically significant conclusions, the analysis of the data may point out a new insight into the spectrum of associated malignant conditions.

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