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Immunoglobulin Sub-typing and Quantification in Direct Antiglobulin Test Positive Haemolysis in an HIV prevalent setting

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ABSTRACT

Introduction: Positive direct antiglobulin tests (DAT) are valuable in identifying the aetiology of autoimmune haemolysis and in guiding therapeutic intervention. However, in human immunodeficiency virus (HIV) positive individuals with background polyclonal gammopathy, a positive DAT in the absence of haemolysis is common. In this setting, immunoglobulin G (IgG) quantification and subtyping may be of value, as this is possible with the recently introduced gel cards. There is paucity of literature evaluating the diagnostic usefulness of IgG subtyping and quantification in HIV positive individuals who are investigated for autoimmune haemolytic anaemia (AIHA). This study evaluated the usefulness of IgG quantification and subtyping in the diagnostic workup of AIHA in patients with a positive DAT, with and without HIV infection.

Methods: This retrospective, cross sectional study included patients investigated for AIHA in a quaternary care hospital. Those with a positive DAT had their IgG subtyped and quantified using the ID – Card DAT IgG1/IgG3 and IgG-dilution cards (Bio-Rad©, Cressier, Switzerland).

Results: Ninety patients admitted from December 2019 to March 2020 were investigated for AIHA. Forty-four (49%) patients had a positive DAT of whom 26 (59%) had evidence of haemolysis, and 16 (36%) were HIV positive. Concurrent HIV and haemolysis were present in eight patients, two of whom had IgG1 although none had an IgG antibody titre >1:30. None of the HIV positive patients without features of haemolysis had IgG1/IgG3 or IgG antibody titres >1:30.

Conclusion: In our clinical setting, IgG quantification and subtyping were found to be of limited value in the diagnostic characterization of AIHA in HIV positive patients with false positive DAT.

INTRODUCTION

Haemolysis is premature or accelerated destruction of red blood cells and anaemia occurs when the rate of red cell breakdown is greater than the red cell production rate by the bone marrow.[1,2] The prevalence of autoimmune haemolytic anaemia (AIHA) in many developing countries is poorly documented. In AIHA, secondary causes can be identified in 50%-70% of all cases, these include drugs, infections, malignancies and autoimmune diseases such as systemic lupus erythematosus (SLE).[1,2]

The Direct Antiglobulin Test and AIHA

In the correct clinical setting, the direct antiglobulin test (DAT) is routinely used to confirm the diagnosis of AIHA. This test detects both red cells coated with immunoglobulin and complement. The clinical utility of this test is dependent on its interpretation in a relevant clinical setting, together with laboratory evidence of red cell breakdown. The 2020 recommendations from the First International Consensus Meeting, for the diagnosis and treatment of autoimmune haemolytic anaemia in adults define the diagnostic criteria required for autoimmune haemolytic anaemia to be the presence of haemolysis, a positive DAT test in the absence of an alternative cause.[2] Conditions characterized by raised immunoglobulin G (IgG) level are associated with nonspecific attachment of IgG to the red blood cells in the absence of haemolysis. These conditions include pregnancy, malignancies, drugs and human immunodeficiency virus (HIV) infection.[3]

HIV infection and AIHA

HIV infection is a common disease in sub-Saharan Africa with an incidence of 2.6/100 person-years reported in the 2011-2015 period.[4] Anaemia is present in 95% of HIV positive patients and is often multifactorial in origin. Haemolysis is considered to be an important cause due to HIV infection or secondary to treatment.[5]

HIV positivity is an independent risk factor for warm AIHA and individuals on combination antiretrovirals (cART) were noted to have a higher risk compared to HIV positive individuals who were not on treatment.[6] Yen et al.[6] reported that HIV positive patients have a 28-fold greater risk for developing AIHA compared to the control group. Proposed causes for this include molecular mimicry, loss of immune tolerance, dysfunctional B lymphocytes and the production of autoreactive antibodies.[6-8]

DAT positivity in HIV infected individuals in the absence of haemolysis is a common occurrence that is hypothesized to be secondary to the HIV infection and/or the treatment.[9-11] Lai et al.[11] showed that DAT positivity, in the absence of overt haemolysis, was associated with lower haemoglobins, treatment with trimethoprim-sulphamethoxazole and co-infection with hepatitis C.[11] In sub-Saharan Africa, with a high prevalence of HIV infection, there is lack of data describing the pattern of DAT results in relation to other clinical features of haemolysis. Better understanding of the pattern of IgG positivity amongst the HIV positive population may translate into a better clinical interpretation of this laboratory finding.

Subtyping IgG and quantifying IgG

The IgG subtypes that are most commonly associated with haemolytic anaemia are IgG1 and IgG3, both of which have increased ability to bind to the Fc receptors on phagocytic cells.[12] Ukita et al.[12] and Dubarry et al.[13] showed positive correlation between the presence of IgG1 and

IgG3 in patients with positive DAT results and clinical/biochemical features of haemolysis. The presence of these IgG subtypes is also proposed to be a predictor of severity of haemolysis.[14-16]

The quantification of IgG in patients with suspected AIHA might help us understand if the amount of IgG attached to the red cell surface is sufficient to cause clinically significant haemolysis.[12,17] In several studies, IgG levels greater than 1:300 were found to correlate with severity of haemolysis.[15,18,19] The gel test for IgG subtyping and quantification is much easier to use and is more cost-effective when compared to flow cytometry, and in fact yields concordant results.[19,20] There is a paucity of literature describing the potential benefit of IgG quantification and subtyping in patients with HIV infection and a positive DAT test.

MATERIALS AND METHODS.

This cross-sectional, retrospective study conducted in a quaternary care hospital was reviewed and approved by the Human Research Ethics Committee of the University of the Witwatersrand. The study period was from December 2019 to March 2020. The study population comprised all patients who were investigated for haemolysis and included all those where IgG was detected on the DAT (DC screening II, card - monospecific anti human globulin reagent for IgG and C3d). The DATs were requested as a part of routine work up in patients with suspected autoimmune haemolysis. Residual blood samples were centrifuged and red cells washed according to the package insert. The washed red cells were then further tested for the presence of IgG1 or IgG3 and quantification of the IgG was performed at 1:10, 1:30, 1:100, 1:300 and 1:1000 dilutions by using ID–Card DAT IgG1/IgG3 and IgG-dilution cards (Bio-Rad©, Cressier, Switzerland). The highest concentration of IgG measured for a single sample was recorded.

For the purpose of this study, haemolysis was defined to be the presence of one or more of the following: reduced haptoglobin, raised reticulocyte count, raised unconjugated bilirubin, raised lactose dehydrogenase (LDH), and/or peripheral smear findings that included spherocytes, polychromasia and nucleated red blood cells. In this study raised biochemical markers of inflammation, namely C-reactive protein (CRP) and/or procalcitonin (PCT) levels were used as surrogate biochemical markers for sepsis. In this study anaemia was defined as haemoglobin concentrations lower than the lower limit of laboratory specific reference ranges for the age and sex of participants. Participants' demographic information was retrieved from the laboratory information system.

Statistical analysis

Qualitative data was described, and quantitative data was tabulated and summarised using standard statistics. Microsoft Excel© (Microsoft Corporation, Washington, USA) and Statistica© (TIBCO Software Corporation, California, USA) programs were used for data collection and analysis of the DAT IgG-dilution and DAT IgG1/IgG3 results.

RESULTS

The demographic data is summarised in Table 1. A total of 90 samples were included in this study, 44 samples with positive IgG on DAT and anaemia, and 46 negative control samples which were confirmed to be IgG negative.

Secondary causes of haemolysis were sought in IgG positive samples with haemolysis (n= 26). These causes included sepsis (13/26, 50%), autoimmune conditions (9/26, 35%) and malignancy (3/26, 12%).

The HIV status was recorded in 34 of the 44 cases with IgG positive anaemia. Of these 34 patients, 16 (47%) were HIV positive and had an IgG positive anaemia (Figure 1). HIV positivity had no statistically significant relationship with warm AIHA (p = .32, 95% CI). In the 16 HIV positive patients with IgG positive DAT tests, the viral loads were available in 15 patients, and CD4 counts in 11. Information regarding cART therapy use was available for four (25%) of these patients. For the HIV positive patients without features of haemolysis there was no information regarding their use of cART. Sepsis was noted in the majority (81%, 13/16) of HIV positive patients with IgG positive anaemia, with and without haemolysis.

Six of the cases with haemolysis demonstrated concentrations of IgG1 (6/26, 23%) (Table 2). In 50% (3/6) of these patients the presence of C3d on the DAT was demonstrated. IgG3 was not detected in any of the 44 patients with and without haemolysis.

Twenty-eight patients in our study (n=90) had concentrations of IgG at ≥ 1:10 (Table 3). Two HIV negative patients with features of haemolysis demonstrated concentrations of IgG at 1:100 and 1:300. The first was an adult female patient with SLE with the highest concentration measured of IgG (1:1000). The second was a paediatric male patient with a malignancy. Both displayed all features of haemolysis and had severe anemia (Hb < 6g/dL).

DISCUSSION

This study highlights the population of patients in a South African setting with DAT positive anaemia. This includes both HIV positive and negative patients, with and without features of haemolysis. The median age of our group of patients (34 years) was lower that of Galic et al.[21] and in the GIMEMA study.[22] This finding may indicate that haemolysis occurs at a

younger median age in a South African setting. The inclusion of paediatric patients (6/44, 14%) in our study is contributory to the younger median age.

In our study there were more females than males in the groups with and without haemolysis. This finding concurs with those reported in the other studies.[14,21-23] Alonso et al.[23] ascribed the increased female: male ratio to the increased susceptibility of females to autoimmune conditions.

Eighty-five percent of the patients with features of haemolysis had secondary warm AIHA. This finding concurs with the studies of Alonso et al.[23] and Galic et al.[21] Two of the patients with warm AIHA had SLE. The top two autoimmune conditions associated with secondary AIHA are SLE and antiphospholipid syndrome.[14,23,24] The majority of patients in our study had features of underlying sepsis regardless of HIV status. This serves as a reminder to clinicians to exclude the presence of associated conditions, such as an undiagnosed malignancy or infections, when there is a positive DAT with features of haemolysis.[14]

Our study population included 16 HIV positive patients (16/34), 47% of those with known HIV status (Figure 1). The presence of non-specific IgG, hyperglobulinaemia and circulating immune complexes attached to red blood cells is often described in the setting of HIV is. Half of the HIV positive patients with IgG positive anaemia did not demonstrate features of haemolysis and would be classified as false positive DAT tests. Our study did not reveal a statistically significant association of HIV infection with haemolysis. Despite this, the appropriate clinical indication to perform specialised laboratory tests, as well as the importance of clinical correlation when interpreting laboratory results, must be emphasised.[11,25]

In all of the HIV positive patients, where the data was available regarding both CD4 count and viral load, had failed to achieve both immunological and virological remission. This is in keeping with the theory of De Angelis et al.[9] and Lepennec et al.[26] who suggested that poorly controlled HIV positive patients were more likely to demonstrate the presence of IgG on the DAT in the absence of haemolysis. The demonstration of IgG in the presence of sepsis and HIV positive patients with no evidence of either virological or immunological remission (12/16, 75%), regardless of the presence of haemolysis, could additionally be an indicator of poor control of the underlying immune deficiency syndrome. Of note, measurements of the CD4 count and viral load assays are mostly freely available and more appropriate for assessing HIV control.

Yen et al.[6] reported that patients on cART, specifically those on Efavirenz, had an increased risk of AIHA. Unfortunately, our study population did not have adequate information regarding cART for the majority of the HIV positive patients included.

In the group of patients with warm AIHA, a small proportion of patients (6/26, 23%) had the subtype IgG1. Galic et al.[21] described similar association of IgG1 positivity in patients with warm AIHA, compared to patients without haemolysis and a positive DAT. The absence of IgG1 in the majority of patients of the patients with haemolysis may be as a result of a different immunoglobulin such as IgA or IgM. Das et al.[14] noted in their study population that the majority of patients with warm AIHA did not demonstrate the presence of either IgG1 or IgG3 and suggested that the presence of either or both of these subclasses could have more value in determining the risk of severe haemolysis in patients.

IgG3 and/or IgG1 were not detected in any of the patients without haemolysis regardless of HIV status. These findings do not agree with those reported by both Lai et al.[18] and Galic et al.[21] of IgG3 detection in cases without haemolysis.

Although IgG1 with or without IgG3 has been reported as commonly associated with AIHA by numerous authors, [15,21,27] correlation of the presence of these IgG subtypes with severity of haemolysis was only described by Das et al.[14] The detection of either or both of these IgG subtypes in patients with suspected AIHA has been proposed to be helpful in stratifying the risk of developing severe haemolysis.[20,24] In our study, IgG1 was only detected in patients with haemolysis. Patients with IgG1 demonstrated variable severity of haemolytic anaemia. The two HIV positive patients with haemolysis and IgG1 were noted to have severe anaemias (Hb < 6g/dL). In HIV positive patients the presence of IgG1 may potentially identify patients with severe AIHA. In addition, the absence of IgG1 in HIV positive patients could suggest there is a false positive DAT test.

In our study, three of the patients, with IgG1 and haemolysis, were positive for C3d on the DAT (n = 26). Two of the three patients were HIV positive, cART naïve, and had viral loads > 100 000 copies/mL with concurrent features of sepsis. The remaining sample was from a HIV negative patient with SLE. Our findings suggest a possible relationship of IgG1 with poorly controlled HIV and autoimmune haemolysis.[9]

A similar proportion of patients with and without AIHA had IgG concentration of 1:10. These low concentrations do not appear to add additional information that may assist clinicians in assessing for the presence of haemolysis. Three patients with warm AIHA had concentrations greater than 1:30 (3/26, 12%). Two of the three patients had an underlying diagnosis of SLE and

severe anaemia. In HIV positive patients with or without haemolysis, none of the concentrations of IgG were greater than 1:30. In our study HIV positive patients, with false positive DAT, quantification of IgG did not yield more useful information.

In conclusion, our study highlights that the demographics and profile of comorbid diseases of South African patients with IgG positive DAT anaemia differs from other studies. The presence of DAT positivity in a patient (especially those with HIV) still requires the correlation of both laboratory and clinical findings. Despite the apparent association between absence of IgG1 and/or IgG3 and the absence of AIHA in HIV positive patients, the overall benefit in both subtyping and quantification in HIV positive patients remains limited.

STUDY LIMITATIONS

This study has the limitations of a retrospective cross sectional study. Our study had a small sample size. Either due to lack of clinical information or haphazard ordering of biochemical tests by treating clinicians, some data points were not available. This further reduced the amount of usable data.

FURTHER RESEARCH

A prospective study in patients with HIV infection, with and without AIHA, to study the presence of IgG1or IgG3 is required to ascertain if subtyping would be of more benefit in this sub-population of patients. Additionally, larger, prospective studies in South African hospitals are encouraged to broaden the information on patients with HIV infection, with and without cART, and positive DAT results.

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CONTRIBUTORS

T Chetty: study design, data collection, data analysis and paper writing; N Bouwer, YO Wan and JN Mahlangu: study design, data analysis, critical review and paper writing. All authors approved the submitted and final version of the manuscript.

COMPETING INTERESTS

Authors declared no conflict of interest with regard to the content of this paper.

KEY MESSAGES

The absence of IG1/IgG3 may be helpful in excluding the presence of severe haemolysis in patients with HIV and a positive DAT.

The subtyping and quantification of IgG in the patients with a positive DAT and HIV has limited value.

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Table 1. Demographic baseline characteristics of the study population

	DAT Positive for IgG (monospecific)		**DAT Negative #**	**Study population**
	With haemolysis	**Without haemolysis**		
Number, n	26	18	46	90
Median age, years	33	40	54	42
DAT results				
IgG	26	18		
C3d	3	0		

Gender				
Female, n	15	15	30	60
Male, n	11	3	16	30
HIV status				
HIV positive, n	8	8	9	25
HIV negative, n	12	6	16	34
Not tested, n	6	4	21	31
Secondary causes				
Autoimmune, n	9	4		
Malignancy, n	3	1		
Sepsis, n	13	9		
Other, n	4	5		
Diagnostic parameters*				
Raised LDH, n	26	-		
Low Hapto, n	17	-		
Raised UBR, n	21	-		
Reticulocytosis, n				

Spherocytosis, n	11	-		
NRBC, n	13	-		
Polychromasia, n	14	-		
	19	-		

Abbreviations:

DAT, direct antiglobulin test; IgG, Immunoglobulin G; C3d, complement by product; HIV, human immunodeficiency virus; LDH, lactate dehydrogenase; Hapto, haptoglobin; UBR, unconjugated bilirubin; NRBC, nucleated red blood cells.

[#]confirmed to be IgG negative.

[*] The diagnostic parameters of the patients determined to have haemolysis.

Table 2. Subtyping of IgG

	IgG positive DAT		**Controls (n=46)**
	Patients with haemolysis (n=26)	**Patients without haemolysis**	
IgG1	6	0	0
IgG3	0	0	0
IgG1 and IgG3	0	0	0

IgG1 and IgG 3 negative	20	18	46
Breakdown of IgG1 positive cases (6): - HIV positive with sepsis (2) - HIV negative with SLE (2) - HIV negative with malignancy (1) - HIV negative with unknown (1)			

Table 3. Quantification of IgG

Dilutions of IgG	**With haemolysis (n=26)**	**Without haemolysis (n=18)**	**Controls (n=46)**
1:10	11	6	0
1:30	6	2	0
1:100/1:300	2	0	0
1:1000	1	0	0

Abbreviations:

IgG, immunoglobulin G