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Role of direct antiglobulin test in assessing the severity of extravascular hemolysis in autoimmune hemolytic anemia – analysis from a cross-sectional study

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ABSTRACT

Background: Autoimmune hemolytic anemia (AIHA) is an immune hemolytic disease characterized by hemolysis and anemia which results from increased red cell destruction due to auto antibodies directed against self-antigens on red cells. Clinical characteristics and potential associated hemolysis in AIHA can greatly depend on the serological characteristics. **Aims and Objectives**: This study was done to find out the association of Grade of polyspecific Direct Antiglobulin test (DAT), complement involvement and immunoglobulin G (IgG) subclass with severity of hemolysis in an AIHA patients. **Materials and Methods**: This was a cross-sectional study conducted for a period of 18 months from March 1, 2012, to August 31, 2013. Fifty consecutive AIHA patients diagnosed by a positive DAT in EDTA anticoagulated blood samples using poly specific anti human globulin reagent were followed up and results of various immunohematological tests such as polyspecific and monospecific DAT, IgG subtyping, and thermal amplitude were performed. Hematological and biochemical parameters such as hemoglobin, percentage of reticulocyte, total serum bilirubin, and serum lactate dehydrogenase also were recorded. Data were analyzed using SPSS v17. **Results**: Majority of patients were belonging to 31–40 year age group and there was a remarkable female predilection. Secondary AIHA was more common (56%) than primary. SLE was identified as the most common underlying disorder in secondary AIHA. Majority (46%) were Grade 4 DAT positive. Predominant autoantibody identified was IgG (52%). Of the total IgG positive cases, IgG1 was the predominant subclass. C3d alone was detected in 18% of cases and C3d and IgG together in 30%. About 48% of our patients were of warm type, 32% mixed type, and 20% cold type. About 80% of the study population were treated with steroids alone. A small percentage of cases (16%) needed immunosuppressant in addition to steroids and still a smaller percentage (4%) needed splenectomy. Higher grades of DAT positivity were associated with more severe degree of anemia and hyperbilirubinemia. The study also demonstrated a positive correlation between DAT grade and severity of hemolysis. **Conclusion:** AIHA patients in this study were predominantly of Warm AIHA with more of IgG1 involvement. A significant association is found between higher grades of DAT results and severity of anemia and jaundice in this study group. A positive correlation exists between grades of DCT and hemolysis as well.

Key words: Auto immune; Hemolysis; Direct antiglobulin; Anemia

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INTRODUCTION

Autoimmune hemolytic anemia (AIHA) is an immune hemolytic disease characterized by hemolysis and anemia which results from increased red cell destruction and/or decreased red cell survival due to auto antibodies directed against self-antigens on red cells.[1] The incidence of disease has been reported to vary from one in 80,000 to 100,000 in a given population per year.[1] Except for countries where malaria is endemic, AIHA is the most common form of acquired hemolytic anemia.[2]

Clinical characteristics and potential associated hemolysis in AIHA greatly depends on the serological characteristics. AIHAs are classically divided into warm and cold auto antibody types based on the temperatures at which these antibodies maximally react with red cells *in vitro*. Warm auto antibodies are more reactive at 37°C than at lower temperatures whereas cold auto antibodies react optimally at 0–5°C and less strongly at higher temperatures. AIHA can occur either as primary (idiopathic) or secondary forms, which are associated with an underlying disorder.[2] In over 80% of patients with AIHA, erythrocyte destruction is extravascular (mainly immunoglobulin G [IgG] mediated) and less frequently intravascular (mainly IgM mediated).[1]

Several factors including quantity, class, subclass and titer of auto antibodies, complement activation, and functional ability of the reticuloendothelial system determine the biological efficacy of the auto antibodies and their ability to provoke *in vivo* hemolysis.[2-4] A rough correlation is said to exist between the amount of antibody fixed to the red blood cell (RBC) surface and the site of destruction, smaller amounts of antibody lead mainly to splenic sequestration and larger amounts of antibody lead to[5,6] increased sequestration within the liver.[8,9] Although IgG alone can mediate RBC clearance, the concomitant presence of RBC-bound C3 (complement) fragments greatly enhances the rate of immune-mediated destruction. The FcγR (Fc gamma receptor), which is the receptor for IgG in reticuloendothelial system have a critical role in immune destruction.[10,11] Of the subclasses of IgG, IgG_3 has the highest affinity for the $F_c\gamma R$ and therefore is most efficient at causing extravascular hemolysis; IgG_3 >IgG_1 > IgG_4 >>>IgG_2.[7,8]

Detection of red-cell-bound Igs and/or complement by direct antiglobulin test (DAT) remains the crucial serological assay in the diagnosis of AIHA.[12] A positive DAT is almost always seen in association with AIHA and forms the hallmark of the diagnosis.[13] However, the mere presence of red-cell-bound Igs does not indicate overt disease, and a combination of clinical and laboratory evidences of hemolysis, such as reticulocytosis, decreased haemoglobin (Hb) and hematocrit, decreased levels of haptoglobin, and increased levels of bilirubin, is required to establish the diagnosis of autoimmune hemolysis.[1] The clinical picture of warm type AIHA constitutes symptoms attributable to anemia and massive hemolysis can be seen. Profound anemia is seen at onset with secondary AIHA.[3,4]

With the advent of Column Agglutination Technology, modifications of DAT testing are available to assess the specific coated component (IgG/c3d/C3b) and subtype of IgG (IgG1/IgG3/others) correlating the hemolytic potential of warm reactive auto antibodies with associated serologic findings mainly strength of DAT and subtyping IgG1/IgG3 may be beneficial because polyspecific/monospecific DAT test is a very simple and cheap test done in all blood centers. Only a few comprehensive studies have been undertaken this aspect across the Indian population and information from India is limited. Hence, the aim of present study is to understand the association and correlation of strength of DAT, involvement of complement, and subtype of IgG with grades of hemolysis produced in an AIHA patient.

Aims and objectives

The aim of the study was to find out the association of Grade of polyspecific DAT, complement involvement and IgG subclass with severity of hemolysis in an AIHA patients.

MATERIALS AND METHODS

Study design

This was a hospital-based cross sectional study done on 50 AIHA patients admitted in the departments of internal medicine and pediatrics.

Study setting

This study was conducted by Department of Transfusion Medicine at Government Medical College, Thiruvananthapuram. The study was done for a period of 18 months from March 1, 2012, to August 31, 2013, and data were compiled and analyzed from September 01, 2013, to January 01, 2014.

Participants

Inclusion criteria

Fifty consecutive AIHA patients visited hematology outpatient department and diagnosed by a positive DAT in EDTA anticoagulated blood samples using poly specific anti human globulin (AHG) reagent.

Exclusion criteria

Cases with associated alloantibody and cases with other hemolytic disorders were excluded along with those who did not express consent.

Sample size calculation

Sample size was calculated assuming a prevalence of 15% for having IgG1 and IgG3 subclass of IgG in AIHA and using the formula $(Z\alpha)^2$ $p \times q/d^2$ where p=15%, q=85% d=10% relative precision and Zα 1.96 sample size was calculated as 51 cases of AIHA.[8]

Sampling

Consecutive sampling of 51 cases was done. Due to missing data, only 50 cases were included in final datasheet.

Ethical considerations

The study was started after getting the clearance from both Human Ethical and Review board. A written informed consent was obtained from all patients included in the study. IRB board approval was obtained from the Institutional Review Board and Human Ethics Committee, Government Medical College Trivandrum HEC No 02/34/2012/MCT dated February 16, 2012. All the procedures adhered to the ethical guidelines of the Declaration of Helsinki.

Variables

Demographic and clinical history of the patients, namely, name, age, gender, permanent address, IP number, name of the Department, clinical features at the time of presentations, diagnosis (primary or secondary), family history, laboratory parameters, and treatment interventions were collected. Independent variable was the grade of DAT by poly specific AHG reagent by Gel method (1+ to 4+, and result of DAT by mono specific AHG anti-Ig (IgG/c3d/both), grades of DAT positivity were further divided into low grade (which included weak +, 1+, 2+) and high grade (which included 3+ and 4+).

Outcome variables were hematological and biochemical parameters such as Hb, percentage of reticulocyte, total serum bilirubin, and serum lactate dehydrogenase (LDH) which were recorded as part of routine investigations. Anemia was considered mild if Hb >7 g/dL and severe if Hb ≤7 g/dL. Moreover, hyperbilirubinemia was taken as mild if total serum bilirubin value ≤2 mg/dL and severe if >2 mg/dL.

The laboratory procedures used in this study were ABO and Rh D blood grouping by tube method, DAT by poly specific AHG reagent by Gel method, DAT by mono specific AHG (anti-IgG and antiC3d) reagent by Gel method, determination of IgG subclasses with anti-IgG1 and anti-IgG3 reagent by Gel method, indirect antiglobulin test (IAT) by Gel method (Antibody screen), thermal amplitude of autoantibodies, and Cross matching test using the Diamed ID-Card ″LISS/Coombs″

DAT by poly specific AHG reagent was done on blood samples of diagnosed and suspected cases using gel technology during the study period and positive cases were further evaluated.

Fresh 5 ml samples were collected into plain and EDTA bottles from each patient. Cell and serum were separated. ABO grouping; both forward and reverse and Rh D typing were done by tube method. All DAT positive cases were further evaluated by gel card impregnated with mono specific AHG reagent (IgG and anti C3d) to classify the auto antibodies into IgG and IgM type. If the antibody was IgG in nature, the subclasses were determined using gel card impregnated with mono specific anti-IgG1 and anti-IgG3. In all the techniques, the agglutination reactions were graded as 4+, 3+, 2+, 1+, weak and negative, and documented accordingly. Those who were positive for DAT were included in the study.

IAT was done on all samples included, by gel method using antibody screening cells. To determine the thermal amplitude of autoantibodies, the patients serum was incubated with 5% of patient cell suspension for 30 min by tube method at three different temperatures; 4°C, 22°C, and 37°C. At the end of incubation period, the reaction mixture in test tubes was checked for agglutination. If there was no agglutination at 30 min, then proceed to an IAT and record the results appropriately. Then, cross-match test was performed by gel method for transfusion purpose and to grade the incompatibility.

Data collection

Details regarding the patient were collected which included name, age, gender, permanent address, IP number, name of the Department, clinical features at the time of presentations, diagnosis (primary or secondary), family history, laboratory parameters, and treatment interventions. These were obtained by an oral questionnaire method, clinical examination, case records. and discussion with the concerned physician. Hematological and biochemical parameters such as Hb, percentage of reticulocyte, total serum bilirubin, and serum LDH were recorded from laboratory data.

Statistical analysis

All statistical data were analyzed using SPSS software version 17. Continuous variables were expressed as mean±standard deviation and qualitative data were expressed as percentage. Categorical variables were compared using Chi-square test. All P-values were two tailed and values of $P<0.05$ was considered statistically significant. Correlations between variables were done using Spearman's correlation test. There were missing data in one case which was removed from analysis. Association between DAT grades and severity of anemia and hyperbilirubinemia was assessed. For this, grades of

DAT positivity were further divided into low grade (which included weak +, 1+, 2+) and high grade (which included 3+ and 4+). In the same manner, severity of anemia and hyperbilirubinemia were also classified into mild and severe forms depending on hemoglobin and total serum bilirubin values. Anemia was considered mild if Hb >7 g/dL and severe if Hb ≤7 g/dL. Moreover, hyperbilirubinemia was taken as mild if total serum bilirubin value ≤2 mg/dL and severe if >2 mg/dL. These parameters were statistically analyzed to find out association by means of Chi-square test.

Correlation between DAT grades and severity of hemolysis was also looked for. For this, *in vivo* hemolysis was categorized into mild, moderate, and severe as per criteria established by previous researchers.[1,13,18] Hemolysis in a patient was documented when any of the following four laboratory parameters were abnormal: (1) Hb (<9 g/dl), (2) reticulocyte count (>2%), (3) total serum bilirubin (>2 mg/dl), and (4) LDH (>500 IU/ml). The hemolysis was classified into mild, moderate and severe on the basis of whether one, two/three, or all the four laboratory parameters were abnormal, respectively.

RESULTS

During the study period, 58 patients presented to the clinic, out of which 55 consented for study. One patient had history of coexisting allo antibodies and one was DAT negative on repeat testing. During follow-up, another patient developed alloantibody, one was not available for follow-up hence excluded from the study. Data were missing for one patient. Hence, a total number of 50 subjects with AIHA were included in the study. Lowest age at diagnosis was 4 months and highest age was 75 years. Most of the cases (46%, n=23) were in the 20–40 year age group. About 22% of patients were minors <18 years (n=11). About 28% (n=14) were between 40 and 60 years of age. Only 2 patients (2%) were >60 years. The mean age was 33 years with a standard deviation of 17.4.

There was a remarkable female predominance in the study population with 72% (n=36) females. About 28% (n=14) of the study subjects were males. Among the total cases, secondary AIHA was more common in occurrence (56% cases, [n=28]) when compared to primary AIHA (44% cases, [n=22]). SLE was identified as the major underlying disorder in 64.29% of the subjects (n=18), rheumatoid arthritis was the underlying cause in 14.29% of the cases (n=4), lymphoma in 10.71% (n=3), AIH in 3.57% (n=1), β Thalassemia in 3.57% (n=1), and DPT vaccination in 3.57% (n=1).

DAT results with monospecific AHG and IgG subtyping results are summarized in Table 1. Distribution of grade/strength of polyspecific DAT obtained is shown in Figure 1.

Outcome of all 50 cases was analyzed. Higher grades of DAT positivity were found to be associated with more severe degree of anemia (Chi-square value 21.809, P<0.05). In the group of patients who had <7 g% Hb at diagnosis (n=31), 27 patients had a high grade (3+ or 4+ DCT) and 4 (12.9%) patients had a lower grade of DAT. In patients who had a Hb >7 g%, (n=19) most of the patients (n=15, 78.9%) showed a low grade DAT (Grade 1 or 2 or weak) and only 4 patients (21.1%) had a high-grade DAT. Odds ratio was 25.3125 with 95% CI 5.5202–116.0692 (P<0.0001).

Higher grades of DAT positivity were also associated with more severe degree of hyperbilirubinemia (Chi-square value 28.288, P=0.001). In 24 patients who had a bilirubin, >2 mg/dl all (100%) had a high grade DAT. In the group

Table 1: DAT test results using mono specific AHG (anti-IgG/antiC3d) and subtyping

Test Parameter	Frequency	Percentage
DAT using mono specific AHG (anti-IgG/antiC3d)		
IgG alone	26	52
C3d alone	9	18
IgG and C3d together	15	30
IgG Subclass		
IgG1 alone	19	46.34
IgG3 alone	2	4.88
Ig G1 and IgG3	7	17.07
Others IgG2/IgG4	13	31.71
Thermal amplitude		
37°C	24	48
4°C	4	8
4°C–22°C	6	12
4°C–37°C	16	32
	50	100

Ig: Immunoglobulin

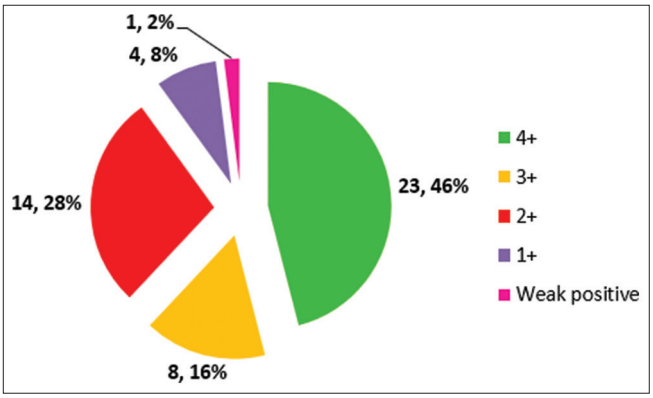

Figure 1: Distribution of grade of polyspecific DAT

of patients who had <2 mg/dl of total hyperbilirubinemia at diagnosis (n=26), 19 (73.1%) patients had a high grade (3+ or 4+ DCT) and 7 (26.9%) patients had a lower grade of DAT.

Pattern of IgG subclass positivity obtained is shown in Figure 2.

IAT done with screening cells (3-cell panel)

All cases showed pan reactivity with screening cells (3-cell panel).

Thermal amplitude of autoantibodies

About 48% of autoantibodies had a thermal amplitude of 37°C only (n=24), 8% (n=4) had a temperature range of 4°C only, 12% (n=6) had a range of 4°C–22°C, and 32% (n=16) had a wide range of 4–37°C.

Laboratory parameters for assessment of hemolysis

The degree of hemolysis in the study population was evaluated based on the following four laboratory parameters, namely, hemoglobin (g/dl), reticulocyte count (%), total serum bilirubin (mg/dl), and LDH (IU/ml). About 50% (n=25) of cases had severe hemolysis, 10% (n=5) had moderate hemolysis, and 24% (n=12) mild hemolysis. Results are shown in Table 2.

Correlation of DAT grades with degree of hemolysis

Hemolysis in a patient was documented when any of the following four laboratory parameters were present: (1) Hb <9 g/dl, (2) Reticulocyte count >2%, (3) Total serum bilirubin >2 mg/dL, and (4) LDH >500 IU/ml. Hemolysis was classified into mild, moderate, and severe on the basis of whether one, two/three, or all the four laboratory parameters were abnormal, respectively. When all the four parameters were normal, it was documented as "no" hemolysis.

Grades of DAT were correlated with degree of hemolysis using Spearman's correlation and were found to have a positive correlation (Table 3) (Spearman's rho Correlation Coefficient r=0.845, P=0.001). Figure 3 displays the linear correlation obtained.

Treatment interventions

Majority of the study population, 80% (n=40) were treated with steroids alone while 16% (n=8) needed immunosuppressive therapy (Azathioprine) in addition to steroids. About 4% of the study group (n=2) needed surgical intervention in the form of splenectomy.

DISCUSSION

DAT is considered to be the hallmark in diagnosis of immune hemolytic anemia and a very simple, quick, and

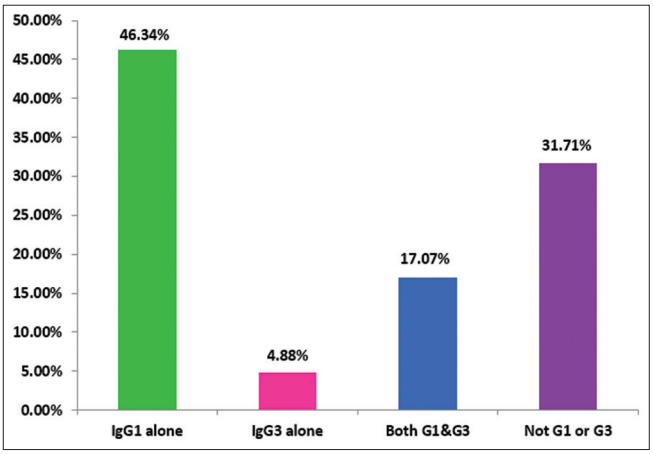

Figure 2: Distribution of study subjects according to IgG subclass positivity

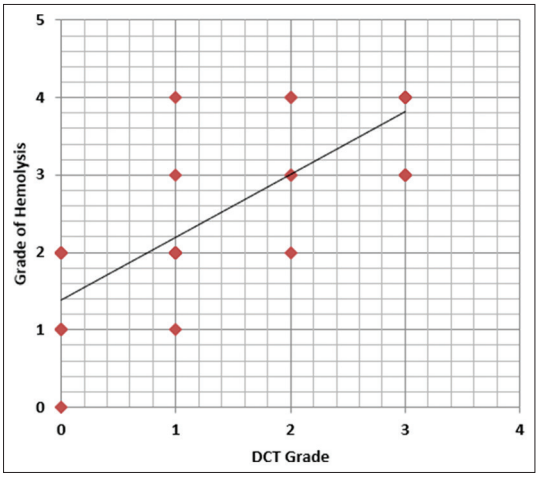

Figure 3: Linear correlation of DAT grades with degree of hemolysis

Table 2: Laboratory indicators of hemolysis

Parameters	Hb (g/dl)	Bilirubin (mg/dl)	Reticulocyte count (%)	LDH (IU/ml)
Lowest value	3	1	1	350
Highest value	10	8	15	4022
Mean	6.8	2.4	5	937.9
Standard deviation	1.9	1.4	3.7	701

LDH: Lactate dehydrogenase

Table 3: Correlation of DAT grades with degree of hemolysis

Degree of hemolysis				Total
No	Mild	Moderate	Severe	
0	1	2	20	23
0	1	2	5	8
4	9	1	0	14
3	1	0	0	4
1	0	0	0	1
8	12	5	25	50

DAT: Direct antiglobulin test

inexpensive test. It is widely used as a qualitative test to know whether the cause of hemolysis is immunogenic or not. However, with the advent of newer techniques using monospecific and IgG subtype specific reagents, it is possible to characterize the involvement of IgG and complement components and also to detect the subtype of IgG. Our study intended to the assess the utility of these additional information in terms of association with grades of autoimmune hemolysis.

Majority of AIHA patients studied were of age between 20 and 40. The study included 11 patients belonging to pediatric and adolescent category also (<17 year). There was a remarkable female predominance in the study population 72% (n=36). Most of our study population were diagnosed as secondary AIHA (56%) associated with SLE. Leading causes of secondary AIHA were reported to be SLE and rheumatoid arthritis[14] and non-Hodgkin's lymphoma.[15] AIHA may precede the onset of lymphomas and connective tissue disorders, and therefore require further investigations and follow-up.

Autoimmune hepatitis is reported to have extrahepatic disease expressions including hemolytic anemia which represented a common factor of disturbed immunological response.[16] Several reports had pointed out that the DPT vaccination also can act as a possible trigger for AIHA.[17] Many case reports were available supporting autoantibody development in β-Thalassemia.[18,19]

The degree of hemolysis in the study population was evaluated based on the following four laboratory parameters, namely, hemoglobin (g/dl), reticulocyte count (%), total serum bilirubin (mg/dl), and LDH (IU/ml).

Based on these four laboratory parameters, the severity of hemolysis was classified into three categories mild, moderate, and severe and 50% (n=25) of cases had severe hemolysis, 10% (n=5) had moderate hemolysis In our study, 16% (n=8) cases were not associated with any hemolysis in spite of being DAT positive. Das et al., classified the severity of hemolysis into two categories – moderate and severe and observed that 39.53% patients had severe hemolysis and 60.47% had moderate hemolysis. In another study from South India involving 175 patients, the severity of hemolysis was classified based on Hb values and found that majority had moderate hemolysis.[20]

Our study group was broadly divided into two groups depending on the grade of DAT positivity; low-grade DCT (38%, n=19) and high-grade DCT (62%, n=31) and association of grade with anemia and jaundice was analyzed separately. A statistically significant association between higher grades of DAT positivity with degree of anemia similarly statistically significant association was observed between higher grades of DAT positivity and degree of hyperbilirubinemia. This finding may help in utilizing DAT as a simple diagnostic tool as well as a predictor for severity and also for monitoring treatment.

There was a statistically significant positive correlation between DAT grade and severity of hemolysis also. Wheeler et al., in their study found that the relationship between the presence or absence of hemolysis and the DAT strength was highly statistically significant.[21]

In the study group, the predominant autoantibody identified was IgG. IgG alone was detected in 52% (n=26) and C3d alone in 18% (n=9). Both IgG and C3d together detected in 30% (n=15) of cases. Several research reports in medical literature described the nature of DAT results in patients with warm AIHA.,[22,23] that is, 20–66% had only IgG on the surface, 24–63% had IgG and C3d, 7–14% had only C3d, and 1–4% cases were DAT negative. On analysing the proportion of IgG, IgM, IgA and complement bound to the RBC surface, 67% had IgG and 32.67% had IgG and C3d.[21] IgG was identified the solitary autoantibody coating the red cells in 72.1% of patients in earlier studies.[20]

Of the total cases associated with IgG positivity, IgG1 was the predominant subclass. In the study group, IgG1 alone was present in 46.3% of cases (n=19), IgG3 alone in 4.88% (n=2). Both IgG1 and IgG3 positivity was seen in 17.07% (n=7). About 31.71% of IgG positive cases (n=13) were not associated with either G1 or G3. Autoantibody showed panreactivity with the antibody screening cells (3-cell panel).

The efficacy of IgG1 and IgG3 to cause hemolysis could not be assessed, because the number of cases associated with IgG3 was very few to statistically analyze.

About 48% of autoantibodies had a thermal amplitude of 37°C only (n=24), 8% (n=4) had a thermal amplitude of 4°C only, 12% (n=6) had a range of 4–22°C, and 32% (n=16) had a wide range of 4–37°C, that is, majority (48%) of autoantibodies were warm type, 32% mixed, and 20% cold type. In a similar study, 81.4% were of warm type, 16.3% of mixed type and there was only one case of cold AIHA (2.32%).[14]

Majority of our study population (80%, [n=40]) was treated with steroids alone while 16% (n=8) needed immunosuppressive therapy (Azathioprine) in addition to steroids and 4% (n=2) needed surgical intervention in the form of splenectomy. Majority of our study population were warm type of AIHA and warm type responded to steroids dramatically. IgG3 was more commonly associated

with severe hemolysis and poor response to the treatment. Patients may have more hemolysis when their red cells are coated with more than one Ig class and subclass compared to single class or subclass.[23]

In our study, population among the eight cases who needed immunosuppressive therapy (Azathioprine), six cases were associated with IgG3. Among this six cases, two cases were associated with IgG3 alone, and four cases were associated with IgG1 and IgG3. In the remaining two cases, one was associated with IgG1 and one with IgG type which was neither G1 nor G3. Among the two cases which needed splenectomy, one was associated with IgG1 alone and the other one with both IgG1 and IgG3. This might explain the more severe clinical picture and poor treatment response in these cases.

Limitations of the study

The study being done on smaller number of patients, scope of statistical analysis in certain parameters such as IgG subclass was limited. Larger multicentric studies are warranted.

CONCLUSION

AIHA patients in this study were predominantly of Warm AIHA with more of IgG1 involvement. A significant association is found between higher grades of DAT results and severity of anemia and jaundice in this study group. A positive correlation exists between grades of DCT and hemolysis as well.

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