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Immunohematological and clinical characterizations of mixed autoimmune hemolytic anemia

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Abstract:

BACKGROUND AND AIM: Patients with warm autoimmune hemolytic anemia (AIHA) may carry immunoglobulin (Ig) M antibodies that react at room temperature and are nonpathological, but few may have cold agglutinins that react at or above 30°C and are referred to as "mixed" AIHA (MAIHA). Here, we present our experience on characterizing MAIHA both clinically and serologically.

MATERIALS AND METHODS: Out of 134 AIHA patients, 13 diagnosed as MAIHA were subjected to detailed immunohematological characterization. Most patients were severely anemic and required urgent transfusions. Resolution of blood group discrepancy, elution, Donath-Landsteiner test, and adsorption study were performed following established protocol. "Best match" blood units were selected and transfused to patients.

RESULTS: Eight of the 13 patients had severe hemolysis. The median age of patient was 37 years with a female preponderance and secondary MAIHA was observed in 8 (61.5%) patients. Blood group discrepancy was encountered in 4 (30.8%) patients. Multiple red cell bound autoantibodies and high titer serum-free IgM autoantibodies were detected in all samples. Twenty-nine units of "best match" packed red blood cells were transfused to 12 patients without any adverse reaction. Improvement in hematological and biochemical values was observed in all follow-up patients.

CONCLUSION: Patients with MAIHA often present with severe hemolysis necessitating blood transfusions. While red cells are coated with multiple autoantibodies, both warm reactive IgG and cold reactive IgM autoantibodies are present in the serum. These serological complexities not only render a crossmatch incompatibility but often lead to blood group discrepancy. "Best match" blood transfusion is always lifesaving.

Keywords:

Autoantibody, autoimmune hemolytic anemia, best match blood, direct antiglobulin test, mixed autoimmune hemolytic anemia

Introduction

Autoimmune hemolytic anemia (AIHA) is characterized by progressive red cell destruction and/or diminished red cell survival caused by antibodies directed against self-antigens on red cells.[1,2] A positive direct antiglobulin test (DAT) is the hallmark of diagnosis. These autoantibodies react at various thermal amplitudes, and accordingly, AIHA is classified as warm, cold, and mixed AIHA (MAIHA).[3,4] While some patients with warm AIHA (WAIHA) may carry immunoglobulin (Ig) M antibodies that react at room temperature and are non-pathological, a few others may have cold agglutinins that react at or above 30 C. The latter are referred to as MAIHA or seldom "combined warm and cold" AIHA. MAIHA can further be subdivided into (a) patients with a high titer, high thermal amplitude IgM cold antibodies and (b) patients with normal titer (<64 at 4°C), high thermal amplitude cold antibodies.[5,6]

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Patients with MAIHA have a chronic course interrupted by severe exacerbations, which can result in severe anemia at times. These exacerbations usually do not appear to be associated with cold exposure, and they do not result in acrocyanosis or Raynaud's phenomenon.[7,8] Whereas DAT is positive for both IgG and complement (C3), most patients show complex serum reactivity in all phases of testing. Red cell elution typically indicates panreactive warm IgG autoantibody. Underlying alloantibodies if any may be detected by adsorption techniques performed at both 37°C and 4°C.[9,10] The cold autoantibody usually exhibits specificity against I antigen, but reactivity against I has also been reported.[5,7,8] Patients generally respond to steroids, and immunosuppressive agents and splenectomy have been employed successful. Severe anemia may need blood transfusion and blood units have to be released as "best match" units due to serological incompatibility caused by the autoantibodies.[1,11] Ours being a referral hospital-based immunohematology facility we receive a request from within and outside laboratories and hospitals for complete characterization of autoantibodies in suspected cases of AIHA. Here, we present our experience on characterizing MAIHA both clinically and serologically with regard to clinical features, antibody class, subclass, DAT strength, and their correlation with *in vivo* hemolysis.

Materials and Methods

The study conducted from July 2013 to August 2016 included 217 patient samples with a provisional diagnosis of "anemia under evaluation" or "AIHA," whereas 134 patients have evidences of *in vivo* hemolysis with a positive DAT, others suffered anemia due to various other causes. These 134 samples were further subjected to DAT evaluation. Samples of 13 patients with a diagnosis of "Mixed AIHA" have been included in the study. A detailed serological characterization was done using gel-based column agglutination technology (CAT) (Bio-Rad, Switzerland). Most patients were severely anemic, had a history of blood transfusions, and few requiring urgent transfusions. For samples showing evidence of autoagglutination, prewarmed technique was used for both red cell and serum study. Any blood group discrepancy was resolved using recommended technique.[9] Samples positive for polyspecific DAT were subjected to monospecific DAT (anti-IgG, anti-IgM, anti-IgA, anti-C3c, and anti-C3d) using dedicated gel cards. For all samples, cold acid elution was done to investigate the type and thermal amplitude of the red cell-coated antibody.[9] To exclude paroxysmal cold hemoglobinuria, the Donath-Landsteiner screening test was performed following established protocol.[9] Serum samples were subjected to reverse grouping, confirmation of free antibodies, thermal amplitudes of antibodies, and adsorption study wherever indicated. Detailed characterization of cold antibodies in serum was performed using conventional tube technique. Polyethylene glycol alloadsorption technique was performed to adsorb free IgG autoantibodies contained in dithiothreitol (DTT)-treated serum to rule out any underlying clinically significant alloantibody.[9,12] To rule out underlying infectious etiology causing AIHA, test for mycoplasma pneumonia, cytomegalovirus (CMV), Epstein–Barr virus (EBV), tuberculosis (TB), treponema pallidum (TP), HIV, and hepatitis-B and C were performed in the microbiology department following specific departmental protocols. Briefly HIV, Hepatitis B and C, CMV, and TP were performed by chemiluminescence assay (ARCHITECT I 1000SR, Abbott Diagnostics, USA). EBV and mycoplasma tests were done by enzyme-linked fluorescent assay (VIDAS, Biomerieux, France) and polymerase chain reaction (PCR) (Biotron Healthcare, India), respectively. TB was ruled out using culture and PCR (Biotron Health care, India) techniques. Rheumatoid factor and antinuclear antibody tests to exclude rheumatoid arthritis and systemic lupus erythematosus were performed in the immunology laboratory using nephelometry (BN ProSpec System, Siemens Healthineers, India) and HEp-2 cell substrate-based indirect immunofluorescence assay. "Best match" blood units were selected by Gel CAT using DTT-treated serum and transfused to patients as per discussed before.[9,11,12] Briefly packed red blood cell (PRBC) units whose reaction strength was found less than that of the autocontrol strength were designated as "best match" units and selected for transfusion.

Results

Of the 134 patient blood samples subjected to complete DAT evaluation, 13 (9.7%) were diagnosed as "Mixed AIHA" clinically and serologically. Warm and cold AIHA constituted 89 (66.4%) and 32 (23.9%) patients, respectively. Clinical and laboratory details of the 13 mixed AIHA patients are presented in Table 1. Eight of the 13 patients had severe hemolysis. The median age of the patient was 37 years (male: 51 years, female: 33 years) with a female preponderance (male: female = 5: 8). Secondary AIHA was observed in 8 (61.5%) patients with underlying lymphoproliferative diseases, collagen diseases, and infections. All patients were severely weak with two patients of severe hemolysis having episodes of passing cola-colored urine. Icterus and organomegaly were observed in 69.2% and 61.5%, respectively. Where the mean hemoglobin (Hb) level of patients was 5.9 g/dl, serum bilirubin and lactate dehydrogenase (LDH) were found to be 3.8 mg/dl and 1470 IU/ml, respectively. Laboratory parameters were significantly deranged in patients with severe hemolysis compared to moderate hemolysis ($P < 0.01$). Immunohematological details of patients are described in Table 2. Blood group discrepancy

Table 1: Clinical and laboratory details of Mixed AIHA patients (*n*=13)

Parameter	Moderate hemolysis (*n*=5)	Severe hemolysis (*n*=8)	Total (*n*=13)
Median Age (range)	33 yrs (19-57)	39 yrs (23-61)	37 yrs (19-61)
Gender (M: F)	2: 3	3:5	5: 8
Disease Duration* (months)			
<12	2 (40%)	4 (50%)	6 (46.2%)
>12	3 (60%)	4 (50%)	7 (53.8%)
Type of AIHA			
Primary	3 (60%)	2 (25%)	5 (38.5%)
Secondary	2 (40%)	6 (75%)	8 (61.5%)
Presenting symptom			
Weakness & fatigue	5 (100%)	8 (100%)	13 (100%)
Palpitation, dyspnoea	2 (40%)	6 (75%)	8 (61.5%)
Yellowish discoloration of eyes	1 (20%)	5 (62.5%)	6 (46.2%)
Fever	1 (20%)	6 (75%)	7 (53.8%)
Abdominal discomfort	2 (40%)	5 (62.5%)	7 (53.8%)
Passing red urine	0	2 (25%)	2 (15.4%)
Physical Signs			
Pallor	5 (100%)	8 (100%)	13 (100%)
Icterus	2 (40%)	7 (87.5%)	9 (69.2%)
Organomegaly	2 (40%)	6 (75%)	8 (61.5%)
Tachycardia/Tachypnoe	3 (60%)/2 (40%)	7 (87.5%)/5 (62.5%)	10 (76.9%)/7 (53.8%)
Laboratory Details (Mean)			
Hemoglobin (g/dL)	6.8	5.3	5.92
Reticulocyte (%)	9.2	14.5	11.3
Serum Bilirubin (mg/dL)	3.1	4.9	3.8
LDH (IU/mL)	1235	1787	1470
IgG class/complement			
IgG + C3c/C3d	5 (100%)	6 (75%)	11 (84.6)
IgG + IgM + C3c/C3d	0	2 (25%)	2 (15.4)

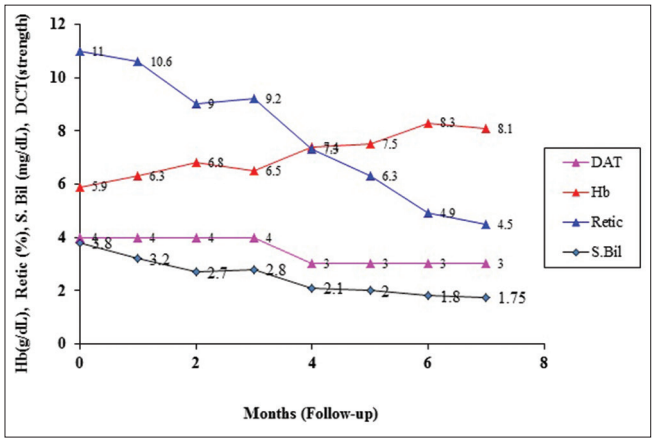

Figure 1: Follow-up of treated "mixed" autoimmune hemolytic anemia patients (*n* = 9)

was encountered in 4 (30.8%) MAIHA patients. Multiple red cell-bound autoantibodies were detected in all the samples with elute expressing panreactive IgG only. For all patients, free serum IgM autoantibodies were reactive at wide thermal amplitude (4°C–37°C) with high titer of IgM and IgG (≥512 and ≥64, respectively). Underlying alloantibody anti-E was detected in one multiple transfused patient. Twenty-nine units of "best match" PRBC units were transfused to 12 patients without any adverse reaction.

A male 51-year-old patient with low agglutinin titer and acceptable Hb of 8.2 g/dl was refused blood transfusion by the treating clinician. In the transfused patient, mean Hb increment was found to be 0.87 g/dL. Of the 13 patients, only 9 (62.2%) revisited regularly for follow-up and all of them were on steroids and immunosuppressant therapy. Improvement in hematological and biochemical values was observed in all patients [Figure 1]. Where no significant reduction in DAT strength was observed in any patients; however, a reduction in cold agglutinin titer (≤1024) was observed after 6 months of therapy.

Discussion

Patients with MAIHA often present with severe hemolysis necessitating blood transfusions. While red cells are coated with multiple autoantibodies, both warm reactive IgG and cold reactive IgM autoantibodies are present in the serum. These serological complexities not only render a crossmatch incompatibility but often lead to blood group discrepancy.

The present study reported 9.7% MAIHA which was marginally higher than the observations of various authors who estimated 6%–8% of mixed AIHA in their patient population.[8,13,14] When categorized etiologically,

Table 2: Immunohematological details of Mixed AIHA patients (*n*=13)

DAT (Polyspecific)	DAT (Monospecific)	Eluate	Titre IgM/IgG	Thermal amplitude of IgM autoagglutinin				Allo-antibody	IgM titre at 6 month follow-up
				4oC	22oC	32oC	37oC		
4+	IgG (3+) C3c (1+) C3d (3+)	IgG (panreactive)	2048/256	4+	4+	2+	1+	None	1024
3+	IgG (2+) C3c (1+) C3d (3+)	Failed	512/64	3+	3+	1+	Neg	None	128
4+	IgG (3+) C3c (1+) C3d (3+)	IgG (panreactive)	1024/512	4+	3+	1+	Neg	None	128
3+	IgG (3+) C3c (1+) C3d (3+)	IgG (panreactive)	512/128	4+	2+	W+	Neg	None	64
4+	IgG (4+) C3c (1+) C3d (3+)	IgG (panreactive)	1024/256	4+	4+	1+	Neg	None	256
4+	IgG (4+) C3c (3+) C3d (3+) IgM (2+)	IgG (panreactive)	4096/1024	4+	4+	2+	Neg	None	512
3+	IgG (2+) C3c (1+) C3d (3+)	IgG (panreactive)	512/64	3+	2+	1+	Neg	None	128
4+	IgG (4+) C3c (1+) C3d (3+) IgM (1+)	IgG (panreactive)	2048/256	4+	4+	2+	1+	None	256
4+	IgG (3+) C3c (1+) C3d (3+)	IgG (panreactive)	1024/256	4+	3+	1+	weak	None	256
4+	IgG (4+) C3c (1+) C3d (3+)	IgG (panreactive)	1024/256	4+	4+	1+	weak	None	128
3+	IgG (2+) C3c (1+) C3d (3+)	Failed	512/64	3+	2+	w+	Neg	None	64
4+	IgG (4+) C3c (3+) C3d (4+)	IgG (panreactive)	2048/1024	4+	4+	2+	Neg	Anti-E	256
4+	IgG (4+) C3c (2+) C3d (4+)	IgG (panreactive)	1024/512	4+	3+	2+	weak	None	64

38.5% and 61.5% of patients had primary and secondary AIHA, respectively, as compared to 55% and 45%, respectively, observed by Sokol *et al*. in a large study of 1834 patients.[15] We observed more cases of secondary AIHA which may be due to symptoms and complications associated with the underlying disorder of secondary AIHA compelling patient to attend hospital. However, more series of studies are needed on this subject as far as the AIHA patient population and demography is concerned and to classify AIHA both on the basis of thermal amplitude and etiology.

The median age of our patient population was 37 years with a female preponderance. Most authors have described high incidence of WAIHA and cold agglutinin syndrome in females above 40 years age.[1,16,17] Such age-incidence data in MAIHA are scarce in the literature. Therefore, multicentric large studies are needed to generate demographic data in MAIHA. However, we assume that, in developing countries including India due to financial and social constraints, middle-aged and elderly patient particularly females are reluctant to visit hospitals and rely on home remedies.

We demonstrated that, in most patients (53.8%), symptoms were slow and insidious over a period of more than 12 months. This finding was in accordance with the study by Pirofsky in 1976 who described majority of patients of AIHA having a chronic waxing and waning course due to the typical etiopathogenesis of the disease.[18] It has been demonstrated earlier that potentially life-threatening presentation is usually associated with viral infection particularly in children.[17]

The present study observed patients with a variable presentation that included weakness, pallor, palpitation, dyspnea, fever, abdominal discomfort, cola color urine, and organomegaly. Such highly variable clinical presentation was also described by others. They observed that, in secondary disease, the symptoms of AIHA may precede the recognition of the underlying illness by months to year, but ultimately symptoms of the underlying disorder dominate.[18-21]

Significant derangement of laboratory values was observed in the patients with bone marrow hyperplasia up to 66%. These may be attributed to severe hemolysis caused by multiple autoantibodies coating the red cells and the free high titer autoantibodies reacting at wide thermal amplitude. The overall impact is a drop in Hb and hematocrit and a rise in reticulocytes, serum bilirubin, and LDH. Wikman *et al*. demonstrated such correlation of low Hb, high LDH, high serum bilirubin, and low haptoglobin with *in vivo* autoimmune hemolysis. They classified hemolysis in to severe when three parameters were deranged, moderate when one or two parameters were deranged, and no hemolysis when all laboratory parameters were within normal range.[21]

Where red cells of all patients were sensitized with IgG and C3, in addition, two patients showed IgM autoantibody coating the red cells. However, the IgM reactivity was low ($\leq$2+), could not be eluted, and could not be detected in the follow-up test after 6 weeks. Previous studies suggested that more effective activation of classical complement pathway is caused by multiple Igs attached to red cells and this has been considered as one of the important causes of increased hemolysis in MAIHA.[22,23] Cold acid elution was performed on all DAT-positive samples with failure in two instances. Panreactive IgG autoantibodies were detected in all elutes tested. All serum samples were subjected to DTT treatment to determine the titer of IgG and IgM autoantibodies separately. A significant high titer (IgM/IgG = 4096/1024) was observed in a 19-year female with underlying lymphoproliferative disease. For all patients, IgM autoagglutin reactivity was observed over wide thermal amplitude with five samples weakly reacting as high at 37°C.

Nine (69.2%) patients visited for follow-up and all of them showed improvement in their hematological and biochemical parameters. The failure of patients to have a subsequent checkup may be the improvement in the clinical signs and symptoms on receiving treatment or the economic constraints restricting them from a second visit. Poor follow-up of primary AIHA patients was also discussed by Prabhu *et al*. in a single-center study.[24] We demonstrated a notable increase in the patient's Hb and a fall in reticulocyte in their follow-up. However, the decrement in the DAT reactivity was poor, and this was due to the fact that corticosteroids primarily act by downmodulating the number of Fc and possibly complement receptors on mononuclear-phagocyte system effector cells, thus improving the survival of the antibody-coated cells without dramatic changes in antibody levels and production.[25] As a secondary phenomenon, the relative amount of antibody per cells decreases as the total number of red cells increase and antibody redistributes. However, long-term corticosteroid and immunosuppressant therapies lead to a significant decrease in autoantibody production.[26] Decision to transfuse in AIHA should be based on the clinical condition of the patient rather than correcting the laboratory values. No critical patient should be denied blood transfusion due to serological incompatibility. However, owing to mixed free autoantibodies in circulation reactive at wide thermal amplitude, it is always advised to observe patients closely during and after blood transfusions.[27,28]

Conclusion

We conclude that detailed clinical and immunohematological characterization is needed to diagnose and manage MAIHA. Whereas corticosteroid and immunosuppressant therapies are the primary therapy, but no critical patient should be denied blood transfusion due to serological complications.

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Conflicts of interest
There are no conflicts of interest.

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