Artículo original

Specificities and isotypes of erythrocytes autoantibodies in patients with warm autoimmune hemolytic anemia

Especificidades e isotipos de autoanticuerpos eritrocitarios en pacientes con anemia hemolítica autoinmune caliente

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ABSTRACT

Introduction: Autoimmune hemolytic anemia is a rare disorder characterized by hemolysis mediated by autoantibodies directed against red blood cells. The demonstration of antibody specificity is a very difficult procedure since autoantibodies in general are nonspecific of antigens and react with all erythrocytes analyzed. Occasionally, specificity is observed against the Rh system antigens.

Objective: To determinate the specificity of erythrocytes autoantibodies in DAT positive autoimmune hemolytic anemia by MAIEA technique.

Methods: The specificity and isotype of erythrocyte autoantibodies were determined in the eluate of 109 blood samples from patients with warm autoimmune hemolytic anemia, by means of the MAIEA technique and the use of monoclonal antibodies that recognized 11 blood group systems and the protein CD47.

Results: In 100% of cases autoantibodies against Rh system antigens were detected; in 24 cases we detected autoantibodies of IgA and IgM isotypes that recognized different antigens that were recognized by IgG isotype autoantibodies.

For idiopathic and secondary warm autoimmune hemolytic anemias, predominance was observed against three or more specificities. IgG was detected in 99.09% of the eluates, IgA in 35.77% and IgM in 16.51%. The high degree of hemolysis was related to the presence of several isotype autoantibodies against four or more blood group specificities.

Conclusions: The MAIEA technique is a sensitive method that can be used to determine the specificities and isotypes of autoantibodies in patients with warm autoimmune hemolytic anemia.

Keywords: autoimmune hemolytic anemia; warm autoimmune hemolytic anemia; monoclonal antibody-specific immobilization of erythrocyte antigens assay; erythrocytes autoantibodie; specificities of erythrocytes autoantibodies; isotypes of erythrocytes autoantibodies.

RESUMEN

Introducción: La anemia hemolítica autoinmune es un trastorno poco común, caracterizado por hemólisis mediada por autoanticuerpos dirigidos contra los glóbulos rojos. La demostración de la especificidad de los anticuerpos es un procedimiento muy difícil, ya que los autoanticuerpos en general no son específicos de los antígenos y reaccionan con todos los eritrocitos analizados. Ocasionalmente, se observa especificidad contra los antígenos del sistema Rh.

Objetivo: Determinar la especificidad de los autoanticuerpos eritrocitarios en pacientes con anemias hemolíticas autoinmunes PAD positivas con el empleo de la técnica MAIEA

Métodos: Se determinó la especificidad e isotipo de los autoanticuerpos eritrocitarios en el eluido de 109 muestras de sangre de pacientes con anemia hemolítica autoinmune caliente, mediante la técnica de MAIEA y el uso de anticuerpos monoclonales que reconocieron 11 sistemas de grupos sanguíneos y la proteína CD47.

Resultados: En el ciento por ciento de los casos se detectaron autoanticuerpos contra los antígenos del sistema Rh. En 24 casos se descubrió autoanticuerpos de isotipos IgA e IgM que reconocieron diferentes antígenos que fueron a su vez reconocidos por autoanticuerpos de isotipo IgG. Se observó para las anemias

hemolíticas autoinmunes calientes idiopáticas y secundarias; predominio frente a tres o más especificidades. Se detectó IgG en el 99,09 % de los eluidos, IgA en 35,77 % e IgM en 16,51 %. El alto grado de hemólisis se relacionó con la presencia de varios isotipos de autoanticuerpos contra cuatro o más especificidades de grupos sanguíneos.

Conclusiones: La técnica MAIEA es un método sensible que puede usarse para determinar las especificidades e isotipos de autoanticuerpos en pacientes con anemia hemolítica autoinmune caliente.

Palabras clave: anemia hemolítica autoinmune; anemia hemolítica autoimmune caliente; técnica de inmovilización de antígenos eritrocitarios con anticuerpos monoclonales (MAIEA); autoanticuerpos eritrocitarios; especificidades de los autoanticuerpos eritrocitarios; isotipos de los autoanticuerpos eritrocitarios.

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Introduction

Autoimmune hemolytic anemia (AIHA) is an uncommon disorder characterized by hemolysis mediated by autoantibodies (AutoAb) directed against self-red blood cells (RBC), with the incidence of 1-3 per 100,000/year and mortality rate is about 11%. AIHA is classified as warm AIHA (WAIHA) (caused mainly by warm-reactive IgG-mediated extravascular hemolysis, comprising 80% to 90% of all AIHA cases), cold AIHA (usually due to complement-mediated intravascular hemolysis, comprising about 15%), and mixed type AIHA (less than 5%), based on the thermal range of autoantibodies involved in the pathogenesis.[1,2]

The presence of positive direct antiglobulin tests (DATs) indicates that RBC AutoAb and/or complement proteins, or both, are bound to the circulating RBCs in vivo. A polyspecific antiglobulin reagent that contains antibodies headed against human IgG and complement proteins (usually C3) is used to screen the patient's RBCs. If positive, monospecific antisera with antibodies to IgG or complement are used to define the pattern of the DAT.[3]

The AutoAb specificities demonstration is a too difficult procedure because the AutoAb generally, are nonspecific antigens and react with all panel RBCs tested. Occasionally, a broad specificity, usually in the Rh system, will be seen. Occasionally, AutoAb specificities toward other blood group antigen systems have been reported. These have included the Kell, Kidd, Duffy, and Diego blood group systems.[4]

The monoclonal antibody-specific immobilization of erythrocyte antigens assay (MAIEA) was initially designed for the assignment of red cell antigens, recognized by human alloantibodies, toward particular components of the red-cell membrane, and has proved to be an effective tool for the investigation of red cell antigens and has been used to study several blood group systems and their associated proteins.[5] This technique has never been used for AutoAb specificities determination in patients with WAIHA.

Keeping in mind that: the identification of the specificity in erythrocytes AutoAb is a hard-working procedure not available in most of the labs; the traditional methods for immunohematological diagnosis do not allow the identification of the specificity on these ones; and the studies involving a great number of cases where specificity is related to severity of the hemolisis have not still been perfomed; we proposed to use the MAIEA technique to determine specificities and isotypes of AutoAb in patients with WAIHA positive DAT.

Methods

Population study

A study of 109 patients with WAIHA positive DAT was carried out. The 71 (65.13%) female and 38 (34.86%) male; idiopathic WAIHA was presented in the 55.96% and 44.03% as secondary to: chronic myelocytic leukemia (n=14), viral infections (n=9), treatment with α-methyldopa (n=4), myelodisplasic syndrome (n=2), systemic lupus erythematous (n=3), multiple myeloma (n=3), chronic lymphocytic leukemia (n=2), lymphoproliferative syndrome (n=2), bone marrow transplant (n=2); acute lymphocytic leukemia, non-Hodgkin lymphoma, liver cancer, colon

cancer, ulcerative colitis, hypothyroiditis and nephropathy; 1 case each. Patients' ages ranged from 1 to 84 years (median: 45 years).

Blood samples and ether elution

After informed consent had been obtained from patients and blood donors, blood samples were collected from venous blood in EDTA (VACUETTE®). The samples were centrifuged at 270 g for 8 minutes at room temperature, and the plasma and buffy coat were removed.

Ether eluates were prepared after washing 1 mL of packed RBCs x 10 using 10 volumes of saline. Elution was performed by adding 1 mL of PBS, pH 7.2 containing 0.4% BSA (Sigma-Aldrich Co.) and 1 mL of diethyl ether (Sigma-Aldrich Co.) to 1 mL of RBCs (an average of 1.1 x 10^{10} RBCs). The mixture was incubated in a 37°C water bath for 10 minutes, with frequent mixing. After centrifugation, the upper layer of ether was discarded and the hemoglobin-stained eluate was transfered into a test tube. The residual ether was evaporated at 37°C for 15 minutes.[6] An average of 1 mL of eluate was recovered. Eluates were kept frozen at –80°C until used.

MAIEA assay (monoclonal antibody-specific immobilization of erythrocyte antigens assay)

Principles and methodology of MAIEA

MAIEA requires the binding of two antibodies, derived from different species, to the same membrane component. The basis for a positive MAIEA reaction is that two antibodies, one a human antibody, the other mouse monoclonal antibody (MoAb), are specific for the same protein, but have epitopes on different regions of the protein. The two antibodies are incubated with a red cell suspension at 37°C for at least 1 h, thus the antibodies are permitted to react with the native protein before the epitopes are potentially denatured during membrane solubilization. The two antibodies and the targeted protein form a trimolecular-complex that is released from the membrane by lysis and solubilization. The

trimolecular-complex is captured by the goat antimouse IgG via the monoclonal antibody and is detected by addition of peroxidase conjugated goat antihuman globulin, which binds to the trimolecular complex via the human antibody. The colored product is measured by the absorbance reading at 492 nm in an ELISA reader. Usually, a ratio greater than 2: 1 is considered positive.[5] In our case the human antibody used is the AutoAb which is present in eluate obtained from the patient with WAIHA.

Precoating of microtitre plates

Microtitre plates (Nunc, Immunoplates) were precoated with 100 μL per well of 1/500 IgG-Fc specific goat anti-mouse (Serotec) in 0.05 M carbonate buffer at pH 9.6. The plate was left overnight at 4 °C and the following morning washed four times with 250 μL PBS/Tween 20 0.05%/Triton X-100 0.5% (PBS-TTw) (Sigma-Aldrich Co.), pH 7.4. The final wash was left for 30 min at 4 °C to block excess binding sites.

Preparation of red lysate membrane component-antibody complex

It was prepared a 10% red cell suspension from 10 blood donors pool of O blood group DAT negative washed three times. 80 μL of this suspension was incubated with 40 μL of mouse MoAb with known specificity toward a membrane component, and 40 μL of eluate containing human AutoAb, for 60 min at 37°C. The sensitized red cells were then washed three times with isotonic saline solution and cell lysis was obtained by the addition of 200 μL of 0.01 M Tris-HCl; 0.15 M NaCl; 2.5 % Triton X-100; containing 2 mM of protease inhibitor PMSF (phenyl-methyl-sulphonyl-fluoride, Sigma-Aldrich Co.). The subsequent lysate was maintained at 4°C for 30 min to permit complete solubilization of the red cell membrane and then centrifuged at 150 g for 15 min to remove the cytoskeleton. 120 μL of each supernatant were diluted with 400 μL of PBS-TTw.

Detection of monoclonal antibody-specific immobilization of erythrocyte antigens

Of the diluted red cell lysates, 100μL were added to the precoatedmicrotitre wells and incubated overnight at 4°C. The plate was then washed four times with PBS-TTw before adding 100μL per well of IgG and IgM Fc-specific peroxidase-conjugated goat anti-human (Serotec), diluted 1/5000; and IgA Fc-specific peroxidase-conjugated goat anti-human (Serotec) diluted 1/2000. The plate was incubated with the peroxidase conjugated antibody for 1 h at 37°C and then washed five times with PBS-TTw.

A substrate solution (100 μL per well) of 0.24 mg/mL of OPD (o-phenylenediamine, Sigma-Aldrich Co.) in 0.05M phosphate citrate buffer, pH 5.0, with 0.024% of hydrogen peroxide (Sigma-Aldrich Co.) was added. The colour reaction was stopped after 15 min by adding 100 μL of 4 N sulphoric acid (Sigma-Aldrich Co.) and the absorbance was read on an ELISA reader (ChemWell 2910) at 492 nm.
Expression of results. The results were expressed as change in absorbance; the difference between the eluate test with those with antibody-negative eluate. Ratios greater than 2.5:1 of absorbance values, was considered in our study.

Identification of specificities and isotypes of human erythrocytes autoantibodies from patients with warm autoimmune hemolytic anemia

For detection of specificities was used the MAIEA technique, described before, with the use of mouse MoAb that recognize human blood group system antigens: anti-CD240 (RhCDE, clone BRIC 69, Serotec, UK), anti-CD239 (Lutheran (LU), clone BRIC 221, Serotec, UK), anti-CD236 (Glycophorin C (Gerbich (GE), clone BRIC 10, Serotec, UK), anti-CD44 (Indian (IN), clone F10-44-2, Serotec, UK), anti-CD55 (Cromer (CROM), clone BRIC 216, Serotec, UK), anti-CD35 (Knops (KN), clone E11, Serotec, UK), anti-CD47, clone BRIC 126, Serotec, UK), anti-CD235a (Glycophorin A (MNS), clone E4, Serotec, UK), anti-Fy3 (Duffy (FY), clone OSK 22,

Osaka Red Cross Blood Center), anti-Band3 (Diego (DI),clone BIII-136, Sigma, USA) and anti-Wr^b (clone CBC-124, Japanese Red Cross Center); diluted 1/20.

The specificities and isotype of human erythrocytes AutoAb were related to idiopathic and secondary autoimmune hemolytic anemia, the hemolysis grade and the presence of more than one immunoglobulin isotype.

The hemolysis grade was divided into two groups: high grade of hemolysis (H1) with Haemoglobin (Hb) <90 g/L, reticulocytes count>5% and haptoglobin level < 0.4 g/L; and low grade of hemolysis (H2) with Hb level between 90-105 g/L, reticulocytes count between 3-5% and normal level of haptoglobin.
In every comparison was used χ^2test, the value $p < 0.05$ was considered statistically significant.

Results

All analyzed cases were detected autoantibodies specificities against antigens of blood groups systems. Autoantibodies against Rh antigens were detectable in the 100% of cases, 10% of these ones did not show other specificities, in the rest, 90% other specificities were detected. The most common no Rh targets were: Band 3, Lutheran, Kell, follows of GPA and Cromer, others blood group proteins in less frequency. The most frequent combinations with Rh specificities were Band 3 in 55 cases (50.46%), of these ones, 30 cases recognized Wr^b antigen; LU in 49 cases (44.95%), K in 38 (34.86%), GPA in 32 (29.36%), Crom in 24 (22.02%), IN 17 (15.60%), CD47 in 10 cases (9.17%) and others specificities in a lesser percentage (Table 1).

Table 1 - Targets of autoantibodies in patients with warm autoimmune hemolytic anemia

Target	No.	%
Rh	109	100
Banda 3	55	50.46
(Wr^b)	(30)	(27.52)
LU	49	44.95
K	38	34.86
GPA	32	29.36
Crom	24	22.02
IN	17	15.6
GPC	12	11
CD47	10	9.17
Kn	5	4.59
Fy	5	4.59

In 22.9% of cases (n=24) we detected autoantibodies of isotypes IgA and IgM which recognized different antigens that were recognized by IgGisotype autoantibodies. In 17 cases we detected the presence of IgA isotype and IgM in 4 cases with different specificities not showed by IgG antibodies. In three patients the specificity change takes place on the IgA as IgM. For both isotypes autoantibodies the most common target was GPA-Wr^b complex which we found in 16 patients (60%) and LU in 7 cases (28%) (Table 2).

Table 2 - Variations of autoantibodies isotypes in patients with warm autoimmune hemolytic anemia

Isotype	Specificities	Other specificities
IgG	IgG,IgA anti Rh,Band3, K	IgA anti GPA,GPC
	IgG anti Rh, K	IgA anti LU
	IgG anti Rh	IgA anti GPA,LU
	IgG, IgA anti Rh, LU, Fy	IgA anti Band3 (Wr^b)
	IgG anti Rh	IgA anti LU
	IgG, IgA anti Rh, IgG anti GPA	IgA anti Band3 (Wr^b)
	IgG, IgM anti Rh, K, IgG anti LU	IgA anti GPA, Crom
	IgG anti Rh, Band3	IgA anti GPA, K
	IgG, IgA anti Rh, IgG anti Band3	IgA anti GPA,K,LU
	IgG, IgA anti Rh, IgG anti Band3,K,Crom	IgA anti GPA,LU
	IgG, IgA anti K, IgG anti Rh,Band3,CD47	IgA anti Kn, LU
	IgG, IgA anti Rh,K, IgG anti Band3	IgA antiGPC, Kn
	IgG anti Rh, Band3, K	IgA anti GPA,
	IgG, IgA anti Rh, IgG anti Band3, K,GPC	IgA anti LU
	IgG, IgA anti anti Rh,Band3, IgG anti LU	IgA anti GPA, GPC, Kn
	IgG anti Rh, GPA, LU	IgA anti Band3 (Wr^b)
	IgG anti Rh, LU	IgA anti GPC, Fy
IgM	IgG, IgM anti Rh, IgG anti GPA, Band3,Crom	IgM anti -IN
	IgG, IgM anti Rh,Band3, IgG anti Wrb, Crom	IgM anti K
	IgG anti Rh, GPA, LU	IgM anti Band3 (Wr^b)
	IgG,IgM anti Fy, IgG anti Rh, LU	IgM anti GPA,CD47,K,Wrb
IgA e IgM	IgG anti Rh, K, Crom, LU	IgA,IgM anti Band3 (Wr^b, IgA anti GPC
	IgG, IgMantiRh, IgG anti GPA,K, IgG, IgA anti GPC, IgG, A, M anti LU	IgA anti Band3 (Wr^b), IgM anti CD47
	IgG, IgA anti K, Crom, LU, IgG anti Rh	IgA anti Band3 (Wr^b),GPC, IgG, IgM anti IN

Idiopathic WAIHA was presented in 56.87% and 43.12% as secondary (previously described), with autoantibodies predominance against three or more specificities for both conditions. We did not find statistically significant differences (p=0.88) (Table 3).

Table 3- Relationship between the number of specificities and idiopathic and secondary conditions

Specificities	Idiopathic		Secondary	
	No.	%	No.	%
Until two specificities	21	19.27	16	14.68
Three specificities or more	41	37.61	31	28.44
Total	62	56.87	47	43,12

χ^2= 0.021; p= 0.88

The IgG was detected in 99.09% of eluates, IgA in 35.77 % and IgM isotype in 16.51%. We just found IgA in a patient. In cases where we detected until three specificities, the predominant isotype was IgG, as others specificities were detected, we showed others immunoglobulin isotypes along with the IgG in 45.84% of cases (p <0.0001) (Table 4).

Table 4 - Pattern of autoantibodies isotypes related to the number of blood group specificities

No. Specificities	IgG No. (%)	IgG + IgA No. (%)	IgG + IgM No. (%)	IgG + IgA + IgM No. (%)
Rh alone	11 (18.33)	0	0	0
Two specificities	19 (31.66)	5(16.66)	1 (12.5)	0
Three specificities	22 (36.66)	7 (23.33)	0	2 (20)
Four or more	8 (13.33)	18 (60)	7 (87.5)	8 (80)
Total	60 (99.98)	30 (99.99)	8 (100)	10 (100)

χ^2= 51.7; p< 0.0001

The high grade of hemolysis was related to the presence of several isotypes autoantibodies against four or more specificities of blood groups (p <0.0001) (Table 5).

Table 5 - Relationship among hemolysis grade, autoantibodies isotypes and the number of blood group specificities

No. Specificities	High grade of hemolysis		Low grade of hemolysis	
	IgG No. (%)	IgG + other isotypes No. (%)	IgG No. (%)	IgG + other isotypes No. (%)
Rh alone	2(1.83%)	0	9(8.25%)	0
Two specificities	10(9.17%)	3(2.75%)	9(8.25%)	3(2.75%)
Until three specificities	2(1.83%)	6(5.50%)	20(18.34%)	3(2.75%)
Four or more	6(5.50%)	22(22.02%)	2(1.83%)	11(10.01%)

$\chi^2 = 54.04$; $p < 0.0001$

Discussion

Is well-known in WAIHA the autoantibodies recognized antigens of Rh blood group system. This affirmation was confirmed with our study. The Rh blood group system, so far itis considered erythroid specific. The assembly of the Rh proteins (RhD, RhCE) and the Rh-associated glycoprotein (RhAG) as a core complex in the RBC and was considered to be a tetramer comprising two molecules of Rh polypeptides and two molecules of RhAG. A recent homology modelling based on crystal structures of bacterial Rh homologues and the human Rh family glycoprotein RhCG, the Rh and RhAG proteins appear to form trimers in the membrane. These ones comprise homotrimers of RhAG and heterotrimers of RhAG with RhD or RhCcEe, but less likely to be heterotrimers of RhAG with RhD and RhCE.[7,8]

RhD, RhCE, and RhAG are part of a macrocomplex of red cell membrane proteins with tetramers of band 3 at its core. Other constituents of this complex are the LW glycoprotein, glycophorins A and B, and CD47, and the complex is linked to

the red cell cytoskeleton through attachment of band 3 along with ankyrin and protein 4.2.[7,8]

The RhD protein provides an opportunity to characterize defects in self-tolerance in different individuals; it represents a target for either auto-aggressive or conventional foreign immune responses. There are a number of different hypotheses to explain the initiation and propagation of autoimmunity in AIHA. Pathology presumably begins with immune recognition of a self-antigen, which may be due to a failure of either central or peripheral tolerance. Such immune dysregulation may be spontaneous, it occurs due to environmental factors such as super-antigens or chronic inflammation, or represents a case of molecular mimicry, in which the immune response toward a foreign antigen (eg. a microbial infection), it cross-reacts with host antigens. Such cross-reactivity may offer amplified levels of particular antigenic sequences, or greater avidity of antigen recognition, to overcome self-tolerance. This molecular mimicry may enhance immunogenicity by innate stimuli.[9,10]

In approximately 90% of studies cases we detected specificities against more thanone specificity. The RBC is one of the most abundance cell types; in these cells we can find 36 blood group systems and low and high incidence antigens series.[11] The RBC is one of the most abundant cell types with major antigenic density and there are very few studies concerning autoantibodies specificities in AIHA. The previous researches demonstrated specificities in less than 50% of cases, using agglutinations techniques and RBC lacking high incidence antigens of blood group systems: LW, GPA, GPC, Banda 3, K, LU, Kidd, Duffy e Indian.[12]

Our findings confirmed the results of Hall et al. This author, in experimental studies of NZB mice genetically engineered proved the lack of the dominant RBC autoantigen Band 3 which has allowed dissection of the underlying mechanisms of spontaneous AIHA. The main results of NZB AIHA´s induction are not dependent on expression of the major autoantigen. In its absence, antibodies are not detected against Band 3, and the corresponding helper response is greatly

attenuated, but, instead, high levels of autoantibodies with other RBC specificities are generated. The immune defect is therefore specific for the RBC type, rather than for the dominant autoantigen. The shift in the specificity of the autoantibody response in these models indicates that the potential for autoaggression is not limited to Band 3, and is consistent with the point of view that a variety of antigens from the target cell may be processed and/or presented in a more immunogenic fashion to drive autoimmune disease. For example, aberrant handling of RBCs by APCs would be a predisposition to autoimmune responses against a range of potential autoantigens. Such a change may arise from activation of APCs, or recruitment of nonprofessional APC types, in response to innate immune signals. Once autoimmunity is initiated, propagation of the response may occur through epitope spreading, ultimately leading to auto-immunization against multiple self-epitopes.[13]

Till we know, there are not previous reports relating to autoantibodies against CD47 protein and Cromer and Knops blood group systems. This is the first notification, but we need more studies to explain the relationship of these autoantibodies and the hemolytic process on humans.

Band 3 was the most frequent specificity found after Rh, that includes Wr^b antigen as specific target in 27.5%. Recent studies described autoantibodies specific for the third loop of Band 3 as a major target in patients with warm antibody AIHA, in accordance with the results of previous studies using immunoprecipitation techniques and suggests that this loop is a preferential target.[14,15]

The Wr^b antigen expression is dependent on the presence of GPA and band 3 and it was suggested that the presence of Glu658 in band3 affects the structure or orientation of the region of GPA between residues 61 and 70 and that the Wr^b antigen is formed when the association between Glu658 of band 3 and GPA presents in this portion of GPA in the correct orientation. Previous reports demonstrated autoantibodies against this antigen in 39% in a series of 30 cases.[16,17] The MAIEA has never been used for this purpose and limits to compare

our results. The main difference may be: a number of investigated cases, the technique used; and RBC used lacking Wr^b antigen that presented alterations on GPA that can masquerade other specificities included inside this glycophorin.

In 24 cases we detected autoantibodies of IgA and IgM isotypes which recognized different antigens that were not recognized by IgG isotype autoantibodies, mainly led against GPA-Wr^b complex and Lutheran glycoprotein. The presence of IgA autoantibodies with different specificities not showed by IgG autoantibodies may be explained by the carbohydrate structures attached to the GPA.[18] The GPA is heavily glycosylated and rich in sialic acid (N-acetyl neuraminic acid), the most abundant red cell sialoglycoprotein with about 15 O-glycans and a single N-glycan; and along with Band 3, the most abundant red cell membrane glycoprotein. GPA is necessary to correct expression of Wr^b antigen and facilitate the translocation of newly synthesized Band 3 toward the membrane.[19] GPA is not essential for band 3 expression at the cell surface, but in red cells deficient in GPA, Band 3 is imperfectly folded and moves slowly to the surface. The Lutheran glycoprotein is member of the Ig superfamily and has five N-glycosylation sites, one in the third domain and the other four in the fourth domain.[20]

Many non-protein antigens, such as polysaccharides and lipids, stimulate antibody production in the absence of helper T cells, and these antigens and the responses they elicit are termed thymus independent or T independent (TI). TI responses may be initiated in the spleen, bone marrow, peritoneal cavity, and mucosal sites. Macrophages located in the marginal zones surrounding lymphoid follicles in the spleen are particularly efficient at trapping polysaccharides when these antigens are injected intravenously. TI antigens may persist for long periods on the surfaces of marginal zone macrophages, where they are recognized by specific B cells.[21]

The most important TI antigens are polysaccharides, glycolipids, and nucleic acids, all of which induce specific antibody production in T cell–deficient animals. These antigens cannot be processed and presented in association with MHC

molecules, and therefore they cannot be recognized by CD4+ helper T cells. Most TI antigens are multivalent, being composed of repeated identical antigenic epitopes. Such multivalent antigens may induce maximal cross-linking of the BCR complex on specific B cells, leading to activation without T cell help requirement. In addition, many polysaccharides activate the complement system by the alternative pathway, generating C3d, which binds to the antigen and is recognized by CR2, thus increasing B cell activation.[22]

On the other hand, cytokines produced by non-T cells may stimulate isotype switching in TI responses. In the absence of T cells, BAFF (B cell-activating factor of the TNF family, also known as BLyS, for B lymphocyte stimulator) and APRIL (a proliferation-inducing ligand, also known asTALL-2, TRDL1 or TNFSF13) produced by cells of myeloid origin, such as dendritic cells and macrophages, can induce the synthesis of AID (activation-induced (cytidine) deaminase) in antigen-activated B cells through a receptor of the BAFF receptor family called TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor). This may be further facilitated by the activation of TLRs on these B cells.[23] In addition, cytokines such as TGF- β that help mediate the IgA switch are secreted by many non lymphoid cells and may contribute to the generation of IgA antibodies headed against non-protein antigens.[24]

The term primary or idiopathic warm AIHA is applied when there is no recognizable underlying or associated condition. In most cases, the ultimate etiology of AIHA is unknown. On the other hand, AIHAs are classified as secondary for several reasons. One reason is the association of AIHA with an underlying disease with a greater frequency compared to others. For example, all authors agree that the incidence of WAIHA is higher in patients with CLL and SLE than in general population. Another criterion is the reversal of the hemolytic anemia simultaneously with the correction of the associated disease. A third reason consists of evidence of immunologic aberration as part of the underlying disorder, especially if the associated disease is thought to have an autoimmune pathogenesis.[12]

The polyclonal immunoglobulin (Ig) class IgG is typically involved in the autoantibody activity of WAIHA, less frequently; it can be associated with IgA and IgM.[1] The results of this study match up with the ones given by other authors for autoantibodies frequency in IgG and IgM classes, but quite different concerning to the IgAone. IgA autoantibodies occur in 15-20% of the patients, either in combination with IgG or, less frequently, alone.[25] The frequency of IgA autoantibodies on patients who were analyzed in this work is higher compared to the ones previously given, it may be due to the immuno-enzymatic test used in the study in order to detect the AutoAb, which matches up with the discoveries previously published by our group.[26]

As far as we know, there have been no series studies on cases that relate the presence of immunoglobulin isotypes with the specificity of autoantibodies in the WAIHA. The results of the study show that the IgA and IgM coexistence with the IgG is related to the presence of autoantibodies against four or more specificities mostly headed against glycophorins and the Wr^b antigen. Concerning this, it has been demonstrated IgM autoantibodies against these antigens on patients with severe WAIHA. *Garratty* et al. described three severe cases (two fatal) of AIHA associated with warm IgM autoantibodies and point out that the specificities of each antibody (En^a, Wr^b and Pr) are all associated with glycophorin A.[27] The severity of AIHA caused by antibodies of these specificities may be related to the role of glycophorin A, an inhibitor of red cell lysis by autologous complement.[28]

The high grade of hemolysis was related to autoantibodies of several isotypes headed againts four or more specificities of blood groups antigens. The previous reports concluded that IgG AutoAb identification along with IgA, IgM, or both immunoglobulins are the main cause of seriousness on autoimmune hemolysis.[26] As we have previously mentioned, our study suggested that the presence of AutoAb of more than one immunoglobulin class on red cells is associated with severe hemolytic anemia. RBC left coated by many different isotopes AutoAb molecules headed against different antigens pertaining to several blood groups systems so it determines the seriousness of hemolytic process.

RBCs sensitized with IgG, IgA and/or sensitized with complement are destroyed predominantly in the spleen and the liver by macrophages. These cells have receptors on their membranes that specifically recognize immunoglobulins, and certain complement components: IgG Fc receptors (FcγR), there are three classes of Fc receptors for this immunoglobulin, and macrophages have all three classes; IgA Fc receptor (FcαR). RBCs sensitized with IgM are removed extravascularly through C3b/iC3b; there are three complement receptors (CR) on macrophages: CR1, CR3 and CR4. CR1 acts as a receptor for C3b, iC3b, C4b and C1q. CR3 mediate phagocytosis and lysis of iC3b-coated RBCs; and is also involved in killing the cells by NK cells. CR4 is closely related to CR3 and also recognizes iC3b.[12,29,30]

Once a macrophage has bound to RBC, it has three ways to effect hemolysis of the RBC:

1) the RBC is engulfed and destroyed inside the macrophage;
2) the RBC is fragmented as the macrophage nibbles fragments out of the RBC membrane, leaving the rest of the RBC and eventually detach and float in the blood, where it can be visualized on a smear as a spherocyte, which may have shortened survival and;
3) the RBC stays outside of the macrophage and is lysed by antibody-dependent cell-mediated cytotoxicity (ADCC) in which the macrophage secretes lytic toxic substances for the attached RBCs.[31

The clinical course of patients with IgA incomplete warm autoantibodies is very similar to the one on patients with IgG antibodies. Destruction of red cells by IgA antibodies may occur by adherence to Fc receptors for IgA on monocytes and macrophages. It has been shown that adherence to this receptor leads to cytotoxic damage or phagocytosis, besides Fcα/μR can also mediate similar responses to IgA and IgM immune complexes.[32]

Commonly, in WAIHA IgM-sensitized RBCs experiment extravascular hemolysis. The explanation is probably the modest activation of the complement pathway, combined with the protective effect of the physiological cell surface complement

inhibitors CD35, CD55 and CD59. CD35 or complement receptor type 1(CR1) has decay-accelerating activity for C3 and C5 convertases of the classical and alternative pathways and work as a cofactor for the factor I-mediated cleavage of C3b and C4b.CD55 or DAF (decay - accelerating factor) protects cells from complement-mediated damage by inhibiting the amplification stage of complement activation. DAF inhibits association and accelerates dissociation of C4b2a and C3bBb, the C3 convertase of the classical and alternative pathways, respectively. Classical pathway C3 convertase regulatory function resides within complement control protein repeats (CCP)-2 and -3, whereas alternative pathway regulatory function resides within CCP-2,3, and 4[33] CD59, also known as the membrane inhibitor of reactive lysis (MIRL), is a complement-regulatory glycoprotein of the Ly-6 superfamily. It inhibits complement-mediated hemolysis by binding to C8 and C9 and preventing assembly of the membrane attack complex.[34,35]

However, it's hard to evaluate the pathogenicity of the IgM AutoAb when these ones come along with the IgG and IgA AutoAb. In the small number of cases when IgM AutoAb have been identified, there are evidences of intravascular hemolysis with a significant mortality which confirms its clinic importance.[36,37]

In essence, the MAIEA technique is a sensitive method that can be used for specificities and isotypes determinations of AutoAb in patients with WAIHA positive DAT. The IgG erythrocytes AutoAb isotype recognized the Rh complex and antigens included in band 3 and glycoprotein Lutheran. However, AutoAb of IgA and IgM isotypes recognize GPA-Wr^b complex more frequently. The high grade of hemolysis was related to AutoAb of several isotypes headed againts four or more specificities of blood groups antigens. This study is the first report concerning to autoantibodies against CD47 protein and Cromer and Knops blood group systems.

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Conflicto de intereses

Los autores declaran que no existe conflicto de intereses.

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Gilberto Soler Noda: Concepción de la idea, realizó diseño general y coordinación del estudio, interpretación de datos, redacción del borrador inicial del artículo, corrección y revisión crítica del manuscrito. Aprobación de versión final.

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