BRIEF REPORT

Defining the clinical relevance of red blood cell autoantibodies by Monocyte Monolayer Assay

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Background: The Monocyte Monolayer Assay (MMA) is an in vitro simulation of red blood cell (RBC) alloantibody behavior. It has been classically applied to predict the risks of post-transfusion hemolytic reactions when transfusing incompatible RBC units. Quantifying erythrophagocytosis by MMA may be an interesting option for situations where there is doubt whether a RBC autoantibody is mediating significant hemolysis. Here, we present three situations involving RBC autoantibodies in which the MMA was decisive for clarifying the diagnosis and choosing the best clinical treatment.

Case Report: *Case 1.* Pregnant patient with severely anemic fetus exhibited warm autoantibody without signs of hemolysis. MMA revealed 30% of monocyte index (MI) highlighting that fetal hemolysis was caused by maternal autoantibody. Prednisone was prescribed with fetal clinical improvement. *Cases 2 and 3.* Two patients with the diagnosis of mixed auto-immune hemolytic anemia and poor response to corticosteroids were evaluated using MMA. The resulting MI was less than 10% in both cases, suggesting that the cold-agglutinin rather than the warm auto-IgG was responsible for overt hemolysis. Treatment with rituximab was begun, with good clinical response.

Conclusion: MMA can be used to evaluate the ability of RBC autoantibodies to mediate overt hemolysis. It can be especially useful to determine the role played by cold and warm auto-antibodies in mixed auto-immune hemolytic disease, helping to define the best treatment option.

KEYWORDS

autoantibody, hemolysis, hemolytic anemia, Monocyte Monolayer Assay

1 | INTRODUCTION

The Monocyte Monolayer Assay (MMA) is a widely known in vitro simulation of the in vivo behavior of anti-erythroid antibodies, whose efficacy has been attested by multiple previous reports.^{1,2} It has been extensively demonstrated that this test is superior to the strength of reaction at antihuman globulin phase and to the IgG subclass in predicting the clinical relevance of RBC alloantibodies.^{1,2}

MMA results are assertive in predicting the risks of symptomatic delayed hemolytic transfusion reactions associated with alloantibodies of known or undetermined specificity, representing the best risk-prediction tool when compatible units are unavailable for transfusion.³ In these situations, relying on literature reports of previous clinical experience to decide about transfusing incompatible units represents a much worse alternative in comparison to performing the MMA.

Predicting the antibody ability to mediate significant erythrophagocytosis may be an interesting choice not only for situations in which an alloantibody of unknown specificity is in scope, but also for situations where there is doubt whether the autoantibody is mediating hemolysis. Here, we present two situations in which the diagnosis of immune hemolysis was doubtful and an *in-house* developed MMA was decisive for clarifying the diagnosis and choosing the best clinical treatment.

2 | METHODS

2.1 | Monocyte isolation and purification

Fifty milliliter of whole blood collected in CPDA-1 bags and originated from male donors were used for the experiments. This blood was further aliquoted in 15 mL samples, which were then centrifuged for 20 minutes at 850 g. The Platelet-Rich Plasma (PRP) was discarded and the cell layer was re-suspended in phosphate-buffered saline (PBS), which was gently deposited on a 15 mL-falcon tube containing 5 mL of Ficoll-Paque™ Plus (GE Health Care, Stockholm, Sweden) in order to isolate the buffy coat layer. The tubes were then centrifuged for 1 hour at 850 g, under 15°C, without break. With the end of this centrifugation step, the buffy coat was collected, washed with PBS three times and re-suspended in RPMI 1640 (Vitrocell Embriolife, Campinas, Brazil) enriched with 10% of fetal bovine serum (Vitrocell Embriolife).

Initially, a pool of macrophages obtained from three male donors was used to perform the assay. After the first experiments, the results obtained using the pool of three donors were the same as those obtained using the buffy coat originated from one single male repeat donor, type O, who was randomly selected among young (<45 years old) and healthy (without clinical comorbidities or drug usage) donors. As the assay requires the concomitant performance of positive and negative controls, and only healthy donors were selected, the risks of misclassifying the clinical relevance of one antibody due to the donor's genetic background (affecting macrophage function) are low.

2.2 | Autoantibody adsorption

Erythrocytes phenotypically identical to the studied patients for the main antigens of Rh system (C, c, E, e); Kell system (K); Kidd system (Jka, Jkb); Duffy system (Fya, Fyb); and MNS system (S,s) were selected for autoantibody adsorption (Case 1) or patients' own sensitized erythrocytes were used in the assays (Cases 2 and 3). 300 μ L of erythrocytes were incubated with 600 μ L of patients' sera during 1 hour at 37°C. The negative control corresponded to the same erythrocytes used in the previous step, but without the incubation with patient's serum. Type O erythrocytes sensitized with commercial anti-D were selected as positive control (Fresenius-Kabi, Friedberg, Germany).

After incubation, erythrocytes were washed three times with PBS. After the last wash, an aliquot of erythrocytes was collected and direct antiglobulin test (DAT) was performed. The remaining RBCs were re-suspended in 900 μ L of RPMI 1640 (Vitrocell Embriolife®).

2.3 | Monocyte Monolayer Assay

Isolated monocytes were seeded on coverslips placed inside a 12-well plate previously coated with 3% cutaneous porcine gelatin (Sigma Aldrich®, St Louis, MO, USA). A mixture of collagen (porcine gelatin) was added to the experiments with the purpose of adhering the coverslips to the plate's wells. The coverslips were later removed and placed over a conventional microscope slide, bypassing the need to acquire specific chambers in order to perform the MMA. This methodology has been previously described for immunofluorescence protocols.^{4,5} Also, there were previous evidences that collagen increased the adherence but did not activate multiple blood cells.⁶ The total amount of monocytes obtained from 50 mL whole blood was suitable for plating four wells. The plate was incubated at 37°C (CO² incubator) for 1 hour. After total adhesion of monocytes, the plate was washed with PBS three times in order to remove non-adhered cells. Sensitized and control erythrocytes (positive and negative) were added to the respective wells, and, then, the plate was incubated for one hour at 37°C (CO² incubator) for the occurrence of phagocytosis. Finally, the plate was washed with PBS three times and the coverslips were stained with Leishman, following previously established protocols. Coverslips were examined microscopically (40 $\times$ magnification).

The Monocyte Index (MI) was calculated as previously described.¹ In brief, a minimum of 200 monocytes per coverslips were evaluated and percentage of those containing erythrocytes inside their cytoplasm or attached to the surface was calculated (MI). Based on previous literature reports evaluating the clinical relevance of RBC alloantibodies, it was stated that MI $\leq$ 5% was associated with little risk of clinical hemolytic reactions; MI between 5.1% and 20% was associated with 33% risk of overt clinical hemolysis and MI >20% was associated with 64% risk of overt clinical hemolysis if incompatible RBC units were transfused. Even though autoantibodies were evaluated in the present study, the same classification was used when interpreting the MMA results.

3 | CASE REPORT

3.1 | Case 1

Intra-uterine transfusion was requested to a 36 year old female, 20th week of pregnancy, with severely anemic fetus. She typed A+ and the irregular antibody screening resulted positive. Identification showed pan-agglutination reaction on all 11 panel erythrocytes, including the auto-control (DG Gel® Coombs, Grifols, Spain). Direct antiglobulin test (DAT) was positive, IgG-only, and the antibody recovered from acid elution agglutinated the entire identification panel. After two auto-adsorptions and one allogeneic adsorption with phenotype compatible erythrocytes, anti-K and anti-E were identified. The patient had no clinical signs of overt auto-immune hemolysis (Hb 11.1 g/dL, Ht 32.9%, VCM 92.4 fL, Lactate dehydrogenase—LDH 213 U/L, reticulocytes 3.72%).

Intra-uterine transfusion was prescribed and compatible RBCs were selected (K- and E-). Fetal pre-transfusion sample revealed

severe anemia (Hb 1.5 g/dL, Ht 4.8%), positive DAT and significant increase in total bilirubin of the amniotic liquid (0.99 mg/dL, reference value <0.1 mg/dL). Acid elution was not performed due to the low amount of erythrocytes in the sample. DNA was extracted from the fetus buffy-coat and genotyped for *RHCE*, *KEL1* and *KEL2* alleles using previously established protocols.⁷ The resulting genotype was *RHCE**ce in homozygosis and *KEL2/KEL2*, excluding any participation of maternal anti-E and anti-K on fetal hemolysis.

Monocyte Monolayer Assay was then performed in order to evaluate the clinical significance of maternal auto-IgG. 1 h-adsorption was performed using mother's serum and selected erythrocytes (K- and E-) in parallel with positive and negative controls as specified in the methods section. Resulting MI was 30%, revealing the autoantibody justified fetal overt hemolytic anemia (Figure 1).

Prednisone 40 mg per day was prescribed to the mother, resulting in progressive improvement of fetal anemia. No more intra-uterine transfusions were required. Labor occurred at 35th week of pregnancy. At birth, the fetus presented a positive DAT with pan-reactive eluate. After a 3-month hospitalization period, the newborn was discharged home without apparent neurologic sequels.

3.2 | Case 2

The sample of a 62 year old male, with the diagnosis of chronic hemolytic anemia of unknown etiology, was sent to our reference laboratory for investigation as the patient had been treated with corticosteroids with poor response. He exhibited moderate anemia (Hb 6.2 g/dL, Ht 19%) and clinical symptoms of fatigue and effort dyspnea. Indirect antibody screening was positive and showed a cold-agglutinin with weak reactivity at 37°C (w- on both screening erythrocytes) and strong reactivity (4+) at 4°C. Direct antiglobulin test (DAT) was strongly positive, revealing IgG, IgM and C3d in the monospecific card. The eluate was reactive on all panel erythrocytes (4+) and persisted so after the treatment with dithiothreitol (DTT). The conclusion was the patient presented both warm auto-IgG and cold-agglutinin over RBC membrane.

In order to define which antibody was majorly mediating patient's hemolysis and properly planning the treatment, MMA was indicated.

The rational was that, if the warm autoantibody was responsible for most of the erythrocyte destruction, MMA would be positive, indicating cell destruction by the reticuloendothelial system. Instead, if the MMA was negative or weakly positive, the main hypothesis would be that intravascular hemolysis mediated by IgM was the main scenario. 1 hour-adsorption at 37°C was performed using patient's serum with both donor compatible and own erythrocytes. The resulting MI was 7%, pointing to the diagnosis of cold auto-immune hemolysis. Rituximab was then prescribed, with clinical improvement.

3.3 | Case 3

A 69 year old female with the diagnosis of Waldenström Macroglobulinemia developed acute hemolysis with severe anemia (Hb 2.2 g/dL, Ht 8%) and poor response to corticosteroids. Her pre-transfusion tests showed strongly positive DAT (4+) with IgG, IgM and C3d on RBCs. Cold-agglutinin reactive at 4°C but non-reactive at room temperature and 37°C was identified in patient's serum. MMA was performed to evaluate whether the warm autoantibody had clinical significance. The MI resulted zero, discarding the autoantibody IgG as the major cause of erythroid destruction. The cold-agglutinin thermic amplitude was then re-evaluated and revealed positive at 22°C, justifying the scenario of intravascular hemolysis complement-mediated deflagrated by cold-agglutinin activation in body extremities. The patient was treated with rituximab, with excellent response (Hb 9.8 g/dL after 1-month treatment).

In this case, the presence of complement covering the autologous RBCs stemmed from IgM binding and complement activation in body parts with lower temperatures. As so, the underlying responsible for both intravascular hemolysis (main mechanism) and complement-mediated extravascular hemolysis (minor mechanism) was the cold-agglutinin. Even though both IgM and IgG antibodies, as well as complement fractions, were detected on the surface of erythroid membrane, MMA was capable of detecting which antibody was the main agent mediating patient's hemolysis. This is justified by the fact that the MMA quantifies only the extra-vascular RBC clearance and, contrary with what happens in the cold-agglutinin-associated

FIGURE 1 Case 1 Monocyte Monolayer Assay result. Adhered monocytes were incubated with selected erythrocytes previously sensitized with the warm autoantibody present in maternal serum (B) in parallel to controls. (A) Monocytes incubated with negative-control erythrocytes. (B) Monocytes incubated with erythrocytes sensitized with maternal warm autoantibody. The resulting monocyte index was 30%

hemolysis, the IgG erythroid destruction is mostly mediated by the reticuloendothelial system and typically associated with high MI.

4 | DISCUSSION

Monocyte monolayer assay is an effective methodology designed to evaluate the ability of anti-erythroid antibodies to mediate extra-vascular hemolysis.³ When compared to other methodologies, either serological or molecular, usually employed by immunohematology reference laboratories, the assay demands similar financial investments, professional expertise and time to conclude the results (Table 1). Even though the MMA main application is to determine the clinical significance of alloantibodies with undetermined specificity or the risks associated with the transfusion of incompatible RBC units when the alloantibody specificity cannot be respected, we here describe situations involving autoantibodies in which this test could be applied, either for diagnostic or therapeutic purposes.

The first situation reported describes a case of severe intra-uterine hemolysis mediated by maternal warm autoantibody. It represents the second description in literature of a maternal auto-IgG causing fetal hemolysis. In the other reported case, the mother also had no signs of clinical hemolysis, but the newborn anemia was mild and no intra-uterine transfusions were required, contrasting with ours.⁸ In our case, the MMA was assertive in indicating that even though the RBC autoantibody was not mediating hemolysis in maternal environment, it could justify immune-mediated erythroid destruction in the fetal reticuloendothelial system. Understanding that two different immune systems

(mother and fetus) could react differently to sensitized erythrocytes and confirming this hypothesis through in vitro simulation was decisive in this case, as it allowed the prompt beginning of immunosuppressive treatment.

The other two cases reported refer to patients diagnosed with mixed auto-immune hemolytic anemia and poor response to corticosteroids. MMA results highlighted in both cases that the cold-agglutinin rather than the warm autoantibody was responsible for erythroid destruction, leading to treatment change and clinical improvement. Our suggestion is that the MMA, as an in vitro simulation of antibody behavior, could be performed when there is doubt whether the RBC autoantibody is responsible for overt hemolytic anemia, mainly when there are both warm and cold autoantibodies covering the erythrocytes. In these situation, a negative or barely positive (MI between 5% and 10%) MMA result would suggest the diagnosis of cold hemolytic anemia. Even though there may exist complement-mediated extravascular erythroid destruction in the case of cold autoantibodies, its contribution to the total hemolysis is significantly inferior to that of intravascular erythroid destruction, justifying the MMA result. Considering that the best therapeutic options differ between cold and warm auto-immune hemolytic disease, defining which antibody is mediating hemolysis saves time and increases the chances of treatment success.

In conclusion, even though the MMA has been classically described as an in vitro simulation of RBC alloantibody behavior, it can be useful to evaluate the ability of RBC autoantibodies in mediating extravascular phagocytosis. MMA can be used to determine the role played by cold and warm autoantibodies in mixed auto-immune hemolytic disease, helping to define the best treatment option.

TABLE 1 Comparison between methodologies commonly applied by Immunohematology Reference Laboratories in terms of costs and difficulty level

	Resolution of immunohematological complex cases using serological techniques		RBC antigen genotyping (single allele)		Monocyte Monolayer Assay	
Inputs/Average cost per patient (US\$)	RBC identification panel	US\$2.00	DNA extraction	US\$3.18	Ficoll-paque	US\$9.00
	Antigen phenotyping	US\$2.50	Taq DNA polymerase	US\$0.81	RPMI culture medium	US\$0.17
	Enzymatic techniques	US\$5.00	DNTPs	US\$1.00	12-wells plastic plate	US\$0.03
			Primers	US\$0.04		
			Agarose gel	US\$1.91		
			Ladder	US\$2.11		
			TEB 1X	US\$0.54		
			Ethidium bromide	US\$0.08		
Average cost per patient (total)		US\$9.5		US\$10.67		US\$9.2
Average Runtime	8 h		8 h		8 h	
Need of specialized employee	Yes		Yes		Yes	

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