Standardization and Evaluation of IgA and IgM Gel Column Agglutination for Direct Antiglobulin Testing

ABSTRACT

BACKGROUND: Diagnosis of autoimmune hemolytic anemia is commonly confirmed with a positive direct antiglobulin test. When standard direct antiglobulin testing is negative and immune mediated hemolysis is suspected, further serological investigation is needed.

STUDY DESIGN: A column agglutination assay for the direct detection of human immunoglobulin A (IgA) and human immunoglobulin M (IgM) was standardized with anti-human IgA and anti-human IgM using recombinant IgA anti-D and monoclonal IgM anti-D. The standardized IgA/IgM detection assay was used to evaluate clinical samples submitted to the immunohematology reference laboratory.

RESULTS: A standardized working dilution of 1:500 was established for anti-human IgA and anti-human IgM in the column agglutination assay. Sixty-one clinical samples were evaluated identifying three reactive samples. These samples included: an IgA and IgM positive sample, an IgA positive sample, and an IgM positive sample.

CONCLUSION: A standardized column agglutination assay for the direct detection of IgA and IgM may be effective when evaluating patients for suspected immune mediated hemolysis when routine direct antiglobulin testing is negative.

Introduction

Autoimmune hemolytic anemia (AIHA) is a relatively uncommon disease process with an incidence between 1 and 3 per 100,000 individuals.[1] Diagnosis of AIHA is commonly confirmed with a positive direct antiglobulin test (DAT). Routine DATs use polyspecific anti-human globulins (AHG) able to detect Immunoglobulin-G (IgG) and complement components (C3b, C3d) on red blood cells. If the polyspecific DAT is positive, monospecific AHGs are used to differentiate if IgG, complement, or both are present on red cells.

The DAT is predictive of suspected AIHA in approximately 83% of patients.[2] However, between 2 and 11 percent of patients that demonstrate clinical or other laboratory signs of AIHA have a negative DAT by routine methods.[3] Technique is important when performing routine direct antiglobulin testing for AIHA. Several reports of DAT negative samples referred to immunohematology reference laboratories demonstrated detectable immunoglobulins by routine methods in over fifty percent of referred samples.[4,5] Three explanations for DAT negative AIHA have been described and include; RBC-bound IgG below the detection threshold of the DAT, Immunoglobulin-A (IgA) and Immunoglobulin-M (IgM) autoantibodies, and low-affinity autoantibodies.[4]

Low affinity antibodies and immunoglobulins below the detection threshold of the routine DAT can be overcome by increasing the sensitivity of the assay. Enhanced techniques such as a LISS wash, direct PEG, Gel DAT, direct polybrene and antibody detection methods with increased sensitivity can be used to identify autoantibodies in routine DAT negative samples.[4,5] Flow cytometry and serological techniques can be used to detect IgA and IgM autoantibodies, however, such methods require standardization and no commercial assays are currently available in the United States[4]. To improve the evaluation of immune mediated hemolysis,

standardization and testing of a laboratory-developed direct antiglobulin test for the detection of IgA and IgM using column agglutination was performed.

Materials/Methods

IgA Sensitized Red Blood Cells

Recombinant IgA1 anti-D (rIgA1 anti-D) (Copenhagen University Hospital, Copenhagen, Denmark) and recombinant IgA2 anti-D (rIgA2 anti-D) (Copenhagen University Hospital, Copenhagen, Denmark) were used as a source to sensitize red blood cells with IgA. Concentrated rIgA1 anti-D and rIgA2 anti-D were diluted to a working concentration in 10% Bovine Serum Albumin (BSA). IgA sensitized red blood cells were prepared by incubating diluted rIgA1/ rIgA2 anti-D with R_1r (DCe/dce) red cells. A 1:1 ratio of packed R_1r cells and diluted rIgA anti-D were incubated for 30' at 37°C and washed four times with blood bank saline. A 0.8% red cell suspension was prepared using the washed packed red cells and MTS™ Diluent 2 (Micro Typing Systems, Inc, Pompano Beach, FL).

IgM Sensitized Red Blood Cells

Commercially prepared IgM anti-D, ALBAclone Anti-D alpha (Quotient Biodiagnostics, Newton, PA), was used as a source to sensitize red blood cells with IgM. Concentrated IgM anti-D was diluted to a working concentration in 10% BSA. IgM sensitized red cells were prepared by incubating diluted IgM anti-D with R_1r red cells. A 1:1 ratio of packed R_1r cells and diluted IgM anti-D were incubated for 15' at 37°C and washed four times with blood bank saline. A 0.8% red cell suspension was prepared using the washed packed red cells and MTS™ Diluent 2.

IgA Antiglobulin

Two sources of anti-human IgA were evaluated.

Rabbit anti-human IgA, α chain specific (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA), was diluted in 10% BSA to a final working dilution.

Goat anti-human IgA, α chain specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was diluted in 10% BSA to a final working dilution.

IgM Antiglobulin

Two sources of anti-human IgM were evaluated.

Rabbit anti-human IgM, $Fc_{5\mu}$ fragment specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was diluted in 10% BSA to a final working dilution.

Goat anti-human IgM, $Fc_{5\mu}$ fragment specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was diluted in 10% BSA to a final working dilution.

DAT by Column Agglutination

Buffered gel cards (Micro Typing Systems, Inc, Pompano Beach, FL) were prepared by adding 25 µl of anti-human IgA or anti-human IgM to a single reaction column and centrifuging the card for approximately five seconds in a MTS™ Centrifuge (Micro Typing Systems, Inc., Pompano Beach, FL). Using the prepared gel card, 50 µl of a 0.8% suspension of washed red cells were added to the appropriate test column and centrifuged for 10 minutes in a MTS™ Centrifuge. Centrifuged gel cards were observed for agglutination and graded following manufacturer's directions[6]. The presence of any agglutination indicated a positive result.

Results

IgA

A checkerboard testing algorithm was performed to determine the optimal working dilution of anti-human IgA. Doubling dilutions of rIgA1 anti-D and rIgA2 anti-D were prepared and used to sensitize R_1r red cells. Increasing dilutions of Goat and Rabbit anti-human IgA were tested with each dilution of IgA sensitized red cells. Samples were tested against 10% BSA as a control for spontaneous agglutination of the sensitized red cells. Rabbit anti-human IgA appeared to be equivalent to Goat anti-human IgA when tested with both rIgA1 anti-D and rIgA2 anti-D (TABLES 1-2). Recombinant IgA1 anti-D demonstrated a higher titer of 1:3200 compared to a titer of 1:100 with rIgA2 anti-D (TABLES 3-4). A working dilution of 1:500 Rabbit anti-human IgA was chosen based on results of initial testing.

Dilutions of 1:3000 and 1:6000 of rIgA1 anti-D were used to sensitize R_1r cells and tested in parallel between column agglutination and flow cytometry. Column agglutination demonstrated agglutination in the 1:3000 sensitized sample and no agglutination in the 1:6000 sensitized sample. Flow cytometry results for both samples were negative. A similar comparison was performed with dilutions of 1:100 and 1:300 of rIgA2. Similar results as with the rIgA1 anti-D were observed with rIgA2 anti-D when compared to flow cytometry testing.

IgM

A checkerboard testing algorithm was performed to determine the optimal working dilution of anti-human IgM. Increasing dilutions of IgM anti-D were prepared and used to sensitize R_1r red cells. Increasing dilutions of Goat and Rabbit anti-human IgM were tested with each dilution of IgM anti-D sensitized red cells (TABLES 5-6). Samples were tested against 10% BSA as a control for spontaneous agglutination of the sensitized red cells. Rabbit anti-human

IgM appeared to be more sensitive than Goat anti-human IgM. A working dilution of 1:500 Rabbit anti-human IgM was chosen based on results of initial testing.

Dilutions of 1:600 and 1:900 of IgM anti-D were used to sensitize R_1r cells and tested in parallel between column agglutination and flow cytometry. Column agglutination demonstrated agglutination in the 1:600 sensitized sample and no agglutination in the 1:900 sensitized sample. Flow cytometry results for both samples were negative.

Clinical Samples

IgM and IgA DATs were performed on samples submitted to the Immunohematology Reference Laboratory. Samples referred for DAT negative workups based on clinical signs and symptoms of hemolysis and samples with identified warm autoantibodies were selected. EDTA anticoagulated samples were chosen and tested within 10 days of collection. Red cells were washed three to four times with blood bank saline then diluted to a 0.8% working red cell suspension using MTS™ Diluent 2. Each sample was tested against Rabbit anti-human IgA, Rabbit anti-human IgM, and 10% BSA in the column agglutination assay. A total of 61 samples were tested (TABLE 7). Three samples resulted in positive column agglutination DAT results (TABLE 8). All three samples were referred as DAT negative workups; however, all had warm autoantibodies identified by routine serological methods. Sample DAT015 demonstrated IgA and IgM on the red cells by column agglutination and IgG and C3 by standard serological methods. Sample DAT026 demonstrated IgM on the red cells by column agglutination and IgG and C3 by standard serological methods. The third sample, DAT028, was submitted for a DAT negative workup and demonstrated IgA on the red cells by column agglutination. This sample reacted with polyspecific anti-human globulin and rabbit anti-human IgG using standard techniques. Both samples reactive with anti-human IgM also had detectable amounts of complement using routine serological methods.

Discussion

Diagnosis of AIHA is routinely confirmed in many clinical and reference laboratories with a positive DAT results. However, when routine testing is negative and immune mediated hemolysis is suspected, additional testing is necessary. Lack of commercially available assays in the United States and literature reports describing standardized testing to detect IgA and IgM coated red cells using column agglutination led to investigation of a laboratory developed test.

This study was designed to develop and standardize a column agglutination test using buffered gel cards and anti-human globulins specific to IgA and IgM. Standardization was achieved using known sources of IgM and IgA and testing dilutions of monoclonal anti-human globulins with specificities to each. Standardization and reproducibility was achieved, however, testing was limited to use of only two examples of recombinant IgA anti-D and one monoclonal example of an IgM anti-D. Comparison near the detection limit of the developed assay with flow cytometry was unsuccessful. Flow cytometry results were negative when compared to positive samples identified in column agglutination testing. This may be suggestive of increased sensitivity, but further investigation needs to be performed to accurately confirm this hypothesis. Additionally, future standardization testing may warrant the inclusion of multiple examples of IgA and IgM antibodies and the use of an alternate testing method or laboratory with the ability to correlate results achieved in the column agglutination assay.

Clinical samples evaluated were a combination of DAT negative referrals and samples with identified warm reactive autoantibodies. These sample populations were chosen to increase the likelihood of identifying an IgM or IgA autoantibody. However, samples submitted for DAT negative evaluations are not necessarily true cases of DAT negative immune hemolysis and may not be the ideal sample population to choose when evaluating the column agglutination assay. Although all samples with positive column agglutination assay results in

this study were referred for DAT negative evaluation, all had detectable IgG and complement on the red cells by standard serological techniques. Earlier reports have identified about 14 percent of warm autoimmune hemolytic anemias as having an IgA component and autoimmune hemolysis attributed to IgA alone to be less than 1 percent of cases[7, 8]. Previously described patient populations with known warm autoantibodies may achieve a better evaluation of the assay in the future and yield more examples of IgA and IgM Autoantibodies[7].

Investigation of a column agglutination assay to detect red cell bound IgA and IgM has led to a successful, reliable and easy to use method of detecting immunoglobulins capable of causing immune mediated hemolysis that may go undetected with standard serological testing methods. Future applications of this assay could include the use of an indirect antiglobulin test to detect serum immunoglobulins not routinely identified by standard serological tests.

References

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		Rabbit anti-human IgA						
		10% BSA	**1:100**	**1:500**	**1:1000**	**1:5000**	**1:10,000**	**1:100,000**
rIgA1 Anti-D	**Neg***	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:100	Neg	4+	4+	4+	4+	4+	3+
	1:200	Neg	4+	4+	4+	4+	4+	2+
	1:400	Neg	4+	4+	4+	3+	3+	+/-
	1:800	Neg	4+	4+	3+	$2+^{s}$	2+	0
	1:1600	Neg	$2+^{s}$	$2+^{s}$	$2+^{s}$	1+	1+	0
	1:3200	Neg	+/-	1+	1+	+/-	Neg	**

TABLE 1. rIgA1 anti-D checkerboard dilution algorithm with Rabbit anti-human IgA. *Non-sensitized red cells. **Not Tested.

		Goat anti-human IgA						
		10% BSA	**1:100**	**1:500**	**1:1000**	**1:5000**	**1:10,000**	**1:100,000**
rIgA1 Anti-D	**Neg***	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:100	Neg	4+	4+	4+	4+	4+	3+
	1:200	Neg	4+	4+	4+	4+	4+	2+
	1:400	Neg	4+	4+	4+	3+^{s}	3+	1+
	1:800	Neg	4+	4+	3+^{s}	3+	2+	0
	1:1600	Neg	2+	2+	2+^{s}	2+	1+^{s}	0
	1:3200	Neg	Neg	1+	1+	Neg	Neg	**

TABLE 2. rIgA1 anti-D checkerboard dilution algorithm with Goat anti-human IgA. *Non-sensitized red cells. **Not Tested.

		Rabbit anti-human IgA			
		10% BSA	**1:100**	**1:500**	**1:1000**
rIgA2 Anti-D	**Neg***	Neg	Neg	Neg	Neg
	1:100	Neg	1+	2+^{s}	2+
	1:200	Neg	Neg	Neg	Neg
	1:400	Neg	Neg	Neg	Neg

TABLE 3. rIgA2 checkerboard dilution algorithm with Rabbit anti-human IgA.
*Non-sensitized red cells.

		Goat anti-human IgA			
		10% BSA	**1:100**	**1:500**	**1:1000**
rIgA2 Anti-D	**Neg***	Neg	Neg	Neg	Neg
	1:100	Neg	+/-	2+	$2+^{s}$
	1:200	Neg	Neg	Neg	Neg

TABLE 4. rIgA2 checkerboard dilution algorithm with Rabbit anti-human IgA.
*Non-sensitized red cells.

		Rabbit anti-human IgM					
		10% BSA	**1:100**	**1:250**	**1:500**	**1:1000**	**1:1500**
IgM Anti-D	**Neg***	Neg	Neg	Neg	Neg	Neg	Neg
	1:100	3+	3+	3+s	4+	4+	4+
	1:200	1+^{s}	1+	3+	3+	3+	3+
	1:300	1+	+/-	1+	1+^{s}	2+	1+
	1:400	Neg	Neg	+/-	1+	1+	1+
	1:500	Neg	Neg	Neg	1+	1+	+/-
	1:600	Neg	**	**	1+	1+	**
	1:700	Neg	**	**	1+	+/-	**
	1:800	Neg	**	**	Neg	Neg	**
	1:900	Neg	**	**	Neg	Neg	**

TABLE 5. IgM anti-D checkerboard dilution algorithm with Rabbit anti-human IgM.
*Nonsensitized cells. **Not tested.

		Goat Anti-Human IgM				
		10% BSA	**1:100**	**1:250**	**1:500**	**1:1000**
IgM Anti-D	**Neg***	Neg	Neg	Neg	Neg	Neg
	1:100	3+	3+	3+s	4+	4+
	1:200	1+^{s}	1+	1+	2+	2+^{s}
	1:300	1+	+/-	+/-	+/-	+/-
	1:400	Neg	Neg	+/-	Neg	Neg
	1:500	Neg	Neg	Neg	Neg	Neg

TABLE 6. IgM checkerboard dilution algorithm with Goat anti-human IgM.
*Non-sensitized red cells.

TABLE 7. Clinical Sample Demographics	
Sample Classification	Number of Samples
Total	61
DAT Negative Workup	46
Warm Autoantibody	15

TABLE 8. Immunoglobulins Detected in Clinical Samples			
Sample	Sample Classification	Immunoglobulins Detected	
		Routine DAT (Anti-IgG, Anti-C3)	GEL DAT (Anti-IgA, Anti-IgM)
DAT015	DAT Negative*	IgG, C3	IgA, IgM
DAT026	DAT Negative*	IgG, C3	IgM
DAT028	DAT Negative*	IgG	IgA

IgM = IgM detected, IgA = IgA detected, C3 = complement detected. *Samples submitted for DAT negative workup.