Efficacy of murine monoclonal antibodies in RBC phenotyping of DAT-positive samples

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Determining the phenotype of patient RBCs that are positive by the DAT may prove problematic. Antigen typing of RBCs coated with IgG requires direct agglutinating reagents or chemical treatment (such as chloroquine diphosphate [CDP] or citric acid) to remove sufficient IgG to permit testing with IAT-reactive reagents. The citric acid elution method is commonly used in the United States; however, antigens in the Kell system are altered to the extent that they may appear to be absent by this method. There are a limited number of direct agglutinating monoclonal antibodies available. Murine monoclonal antibodies provide an additional tool for typing RBCs with a positive DAT. Five murine monoclonal IgG antibodies (anti-K: MIMA-22, MIMA-23; anti-Kp^a: MIMA-21, MIMA-27; anti-Fy^a: MIMA-19) were used in this study. Donor RBCs with known phenotypes were sensitized in vitro with alloanti-D, alloanti-c, and alloanti-K and with 20 autoantibodies (autoanti-D [n=3], autoanti-e [n=5], autoanti-Ce/e [n=5], autoanti-e+D+E [n=1], autoanti-I [n=1], and nonspecific [n=5]) to simulate a positive in vivo DAT. The sensitized RBCs were treated with CDP to remove IgG. To determine the efficacy of the murine monoclonal antibodies when testing DAT-positive samples, both sensitized and CDP-treated RBCs were tested with these monoclonal antibodies by the IAT using anti-mouse IgG. No discrepancies were noted with the unsensitized, sensitized, or CDP-treated RBCs. An exception was noted with a potent autoanti-I, where direct agglutination of the sensitized RBCs was obtained. This study demonstrates the value of using murine monoclonal antibodies to determine the phenotype of RBCs with a positive DAT caused by autoantibodies (e.g., in autoimmune hemolytic anemia) and supports previous studies showing that RBCs sensitized in vivo can be typed without chemical manipulation. ***Immunohematology* 2006;22:161–165.**

Keys Words: blood groups, murine monoclonal antibodies, anti-mouse IgG, autoantibodies, DAT, chloroquine diphosphate

Determining an accurate RBC phenotype for patients whose RBCs are positive by the DAT can be problematic because these RBCs are already coated in vivo with immunoglobulin, complement, or both; all tests performed will be positive by IAT with antihuman IgG reagents. There are very few IgM directly agglutinating reagents available for the clinically significant antibodies (i.e., anti-K, -Jk^a, -Jk^b, -S, and -Fy^a). One option is to chemically treat the DAT-positive RBCs to remove IgG autoantibodies. These treatments may use chloroquine diphosphate (CDP), a combination enzyme/reducing agent (ZZAP, National Blood Service Reagents, Birmingham, UK) which is composed of DTT and papain, or EDTA/citric acid (Elu-Kit II, Gamma Biologicals, Inc., Houston, TX). Several other methods are available to remove the antibody for serologic testing; however the RBCs are not suitable for testing after these treatments.[1–5]

CDP and enzyme/reducing agent (ZZAP, National Blood Service Reagents) treatments can cause damage to the RBCs, resulting in the loss of some RBC antigens and possible invalid typing results.[6–8] Additionally, CDP may not totally remove the coating autoantibody from the RBCs and it does not remove complement component 3 (C3).[1] The proteolytic enzyme, papain, in the enzyme/reducing agent product denatures some MNS and Duffy antigens. The reducing agent, DTT, denatures the antigens in Kell and Yt systems among others. RBCs treated with the reagent combining both these chemicals (ZZAP, National Blood Service Reagents), therefore, have limited applications for use in phenotyping studies.[9] The citric acid elution method is commonly used in the United States, a major drawback being that antigens of the Kell system are significantly weakened by this method. The development of murine monoclonal antibodies has provided an additional tool to allow DAT-positive RBCs to be antigen typed.[10,11]

Materials and Methods

Selected patient samples containing alloantibodies and autoantibodies were obtained from the samples routinely referred to the Red Cell Immunohaematology Laboratory, National Blood Service, Colindale Centre, UK. Samples from three antenatal women with high titers of anti-D, -c, and –K, respectively, were selected for testing. A total of 20 autoantibodies from patients

with autoimmune hemolytic anemia (AIHA) were also selected: anti-D (n=3), anti-e (n=5), anti-Ce/e (n=5), anti-e+D+E (n=1), anti-I (n=1), and nonspecific (n=5). (See Table 1 as an example of phenotyping results using murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs for sample 2). All samples were collected in EDTA and were tested within 5 days of collection. The murine monoclonal antibodies used included MIMA-19: (anti-Fy^a), MIMA-21: (anti-Kp^a), MIMA-22: (anti-K), MIMA-23: (anti-K), and MIMA-27: (anti-Kp^a). Ten different commercial human monoclonal antibodies were also tested; anti-C, -c, -E, and -e (MS-24, MS-33, MS-258/906, MS-16/MS-21/MS-63 respectively, Biotest AG, Dreiech, Germany); anti-C^w, -Jk^a, -Jk^b, and -S (MS-110, MS-15, MS-8, MS-94 respectively, Serologicals Ltd., Livingston, Scotland), anti-K (MS-56, Lorne Laboratories, Reading, UK), and anti-Fy^a (5T72, Bio-Rad, Marines La Coquette, France).

Sensitization of donor RBCs

Aliquots of 500 µL of washed, packed RBCs from nine donors (R_1R_1 [n=3], R_2R_2 [n=3], and rr [n=3]) were each incubated with 500 µL of the alloantibody or the autoantibody at 37°C for 60 minutes. Sensitized RBCs were confirmed by obtaining a positive DAT using polyspecific antihuman reagent (AHG, Lorne Laboratories).

CDP treatment

CDP (200 mg/mL, pH 5.0 ± 0.1, National Blood Service Reagents) was used to treat RBCs with a positive DAT. Four volumes of CDP were added to 1 volume of washed (×4) DAT-positive packed RBCs, mixed, and incubated at 20°C for a maximum of 2 hours. The strength of the DAT was tested after a 30-minute incubation at 37°C using a monospecific antihuman reagent (anti-IgG, Biotest AG).

Hemagglutination testing

All of the treated RBC samples were washed 4 times with 0.9% PBS and resuspended in a low ionic strength solution (LISS, Inverclyde Biologicals, Bellshill, Scotland) at a 2% suspension. Both treated and untreated RBCs were tested with murine monoclonal antibodies by IAT (40 µL:40 µL) and incubated at 20°C for 30 minutes using a sheep-derived IgG reagent directed at mouse IgG (sheep-anti-mouse IgG, The Binding Site, Birmingham, UK, code PC271.X). The sheep-derived reagent was diluted at a ratio of 1 in 50 in a bovine serum albumin solution (1% BSA, Serologicals, Kankakee, IL) in PBS.

Table 1. An example of phenotype results of murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs (Sample 2)*

Sample 2 Alloanti-K	Donor's Phenoytpe				Treated or Untreated RBCs	Anti-K (MS56) Lorne	Anti-Fy^a (5T72) Bio-Rad	Anti-Fy^a MIMA-19	Anti-Kp^a MIMA-21	Anti-K MIMA-22	Anti-K MIMA-23	Anti-Kp^a MIMA-27	*DAT*
	Rh	K	Fy^a	Kp^a									
Donor 1	R_1R_1	+	+	0	Unsensitized	4	4	4	0	4	NT	0	***0***
					Sensitized	5	4	3	0	3	NT	0	***4***
					CDP treated	5	4	3	0	4	NT	0	***0***
Donor 2	R_1R_1	+	+	0	Unsensitized	4	4	3	0	5	NT	0	***0***
					Sensitized	5	4	3	0	3	NT	0	***4***
					CDP treated	5	2	3	0	4	NT	0	***0***
Donor 3	R_1R_1	+	0	0	Unsensitized	4	0	0	0	5	NT	0	***0***
					Sensitized	5	**4**	0	0	3	NT	0	***4***
					CDP treated	5	0	0	0	3	NT	0	***0***
Donor 4	rr	+	0	0	Unsensitized	4	0	0	0	5	NT	0	***0***
					Sensitized	5	**4**	0	0	3	NT	0	***4***
					CDP treated	5	0	0	0	4	NT	0	***0***
Donor 5	rr	+	+	0	Unsensitized	4	3	3	0	5	NT	0	***0***
					Sensitized	5	4	2	0	3	NT	0	***4***
					CDP treated	5	4	3	0	4	NT	0	***0***
Donor 6	rr	+	+	0	Unsensitized	4	4	3	0	5	NT	0	***0***
					Sensitized	5	4	2	0	3	NT	0	***4***
					CDP treated	5	4	3	0	4	NT	0	***0***
Donor 7	R_2R_2	+	+	0	Unsensitized	4	4	4	0	5	NT	0	***0***
					Sensitized	5	4	3	0	3	NT	0	***4***
					CDP treated	5	3	3	0	4	NT	0	***0***
Donor 8	R_2R_2	+	+	0	Unsensitized	4	4	3	0	5	NT	0	***0***
					Sensitized	5	4	3	0	3	NT	0	***4***
					CDP treated	5	3	3	0	4	NT	0	***0***
Donor 9	R_2R_2	+	+	0	Unsensitized	4	3	3	0	5	NT	0	***0***
					Sensitized	5	4	3	0	3	NT	0	***4***
					CDP treated	5	3	3	0	4	NT	0	***0***

*Grading per system for hemagglutination used by Red Cell Immunohaematology Laboratory, National Blood Service, Colindale Centre, UK

- 5 Cell button remains in one clump or dislodges into a few large clumps, macroscopically visible
- 4 Cell button dislodges into numerous large clumps, macroscopically visible
- 3 Cell button dislodges into many clumps, macroscopically visible
- 2 Cell button dislodges into finely granular but definite small clumps, macroscopically visible
- 1 Cell button dislodges into very small agglutinates; these are more distinct if the tube is left on its side to allow the cells to settle, macroscopically visible
- < 1 Grainy appearance, agglutinates best seen microscopically
- 0 Negative result

Adsorption and elution

Two mL of donor RBCs were washed 3 times in PBS, added to 2 mL of sensitizing antibodies, and incubated at 37°C for 60 minutes. To ensure sensitization, a DAT was performed on the sensitized RBCs with polyspecific antihuman reagent (AHG, Lorne Laboratories). An eluate was prepared using an EDTA/citric acid kit (Elu-Kit II, Gamma Biologicals, Inc.). The eluate and last wash were each tested by IAT.

Validation by flow cytometry

The five murine monoclonal antibodies (MIMA-19, MIMA-21, MIMA-22, MIMA-23, and MIMA-27) were validated by flow cytometry. To validate the tests, 4 µL of packed unsensitized, sensitized (DAT-positive), or CDP-treated RBCs were added to 40 µL of MIMA-19, MIMA-23, MIMA-21, MIMA-22, and MIMA-27 in plastic tubes. After 30-minute incubation in a 37°C water bath, the tubes were washed 3 times with 0.9% PBS. Fifty µL of FITC-conjugated $F(ab')_2$ goat-derived reagent directed at mouse immunoglobulin (goat anti-mouse immunoglobulin DAKO A/S, Denmark, code F0479) was added and the tubes were incubated for 30 minutes at 37°C in the dark. The mixture was washed twice and resuspended in 1% BSA in PBS. The tubes were analyzed by a flow cytometer (FACSCAN, Becton Dickinson, Oxford, UK)

Results

Adsorption and elution validation demonstrated autoantibodies that were successfully bound to the donor RBCs and could be eluted off. Flow cytometry results confirmed the selected murine monoclonal antibodies bound specifically to target antigens with unsensitized, sensitized, and CDP-treated RBCs. (See Figures 1a-c.) No nonspecific binding was observed with antigen-negative donor RBCs. (Results not shown).

Table 1 shows the phenotyping results of sample 2 (allo anti-K) using murine monoclonal antibodies with unsensitized, sensitized, and CDP-treated RBCs from nine donors (3 × rr, 3 × R_1R_1, and 3 × R_2R_2). Post sensitization, all of the donor's RBCs were positive by the DAT which confirmed that the donors' RBCs were coated with the sensitizing antibody in this study. No discrepant results were observed with the murine monoclonal antibodies tested. However, there was insufficient anti-K (MIMA-23) for testing samples number 1 to 3. The false positive reactions with the RBCs from donors 3 and 4 using IgG monoclonal

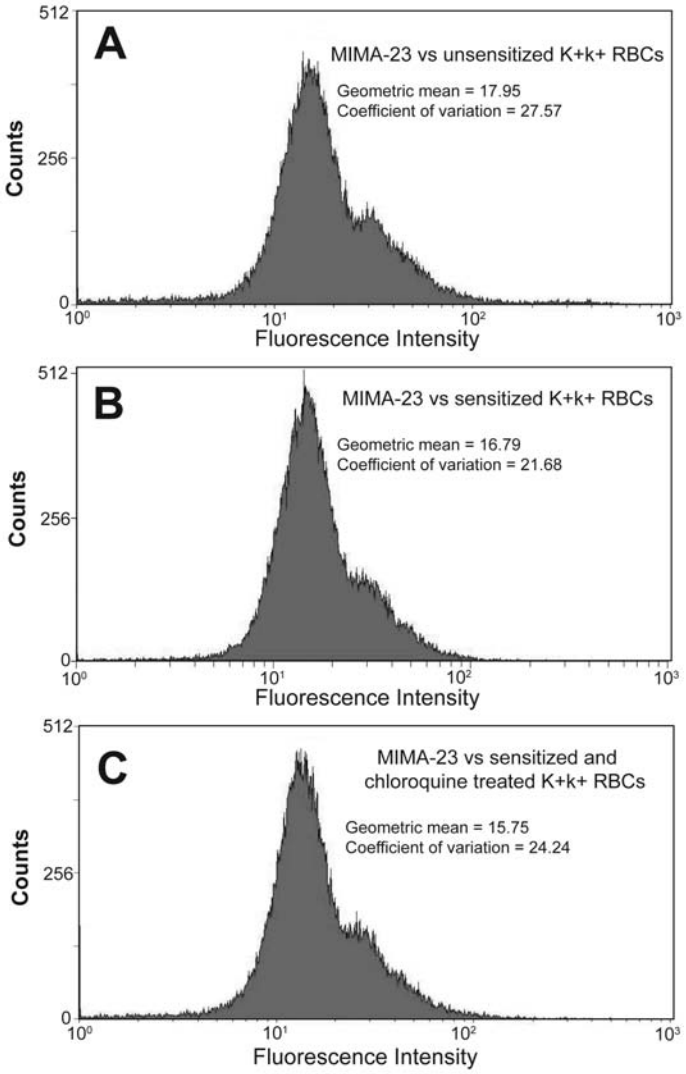

Fig. 1. Binding of MIMA-23 (anti-K) with unsensitized, sensitized, and CDP-treated K+k+ RBCs. **(A)** MIMA-23 versus unsensitized K+k+ RBCs; **(B)** MIMA-23 versus sensitized K+k+ RBCs; **(C)** MIMA-23 versus. sensitized and chloroquine-treated K+k+ RBCs.

IAT-reactive anti-Fy^a were not surprising. As the postsensitized donor RBCs were DAT positive, a false positive result would be expected by IAT.

A summary of the phenotyping results for all antibodies tested is shown in Table 2 (actual results not shown). There were no discrepant results obtained with the murine monoclonal antibodies used; however, false positive reactions were obtained with commercial anti-sera and are shown in the Results column.

The test results with unsensitized, sensitized, and CDP-treated RBCs were concordant with one exception: a potent autoanti-I caused direct agglutination of all RBC samples.

Table 2. Summary of tested results (n=23)

Sample ID	Allo or autoantibody specificity	Result
1	Alloanti-c	False positive with commercial anti-K and -Fy^a
2	Alloanti-K	False positive with commercial anti-Fy^a
3	Alloanti-D	False positive with commercial anti-Fy^a
4	Autoanti-e	False positive with commercial anti-Fy^a and -Jk^b
5	Autoanti-e	No discrepancies
6	Autoanti-D	False positive with commercial anti-Fy^a
7	Autoanti-Ce/e	False positive with commercial anti-Fy^a, anti-c, and anti-E
8	Autoanti-e	False positive with commercial anti-Fy^a
9	Autoanti-D	False positive with commercial anti-Fy^a
10	Autoanti-e	No discrepancies
11	Autoanti-e+D+E	False positive with commercial anti-Fy^a + S
12	Autoanti-Ce/e	No discrepancies
13	Autoanti-Ce/e	No discrepancies
14	Autoanti-Ce/e	No discrepancies
15	Nonspecific autoantibody	False positive with commercial anti-Fy^a, anti-Jk^a, and anti-Jk^b
16	Nonspecific autoantibody	False negative with commercial anti-Jk^b
17	Nonspecific autoantibody	No discrepancies
18	Autoanti-D	False positive with commercial anti-Fy^a
19	Nonspecific autoantibody	False positive with commercial anti-Fy^a and anti-Jk^b
20	Nonspecific autoantibody	False positive with commercial anti-Fy^a and anti-Jk^b
21	Autoanti-I	Positive with all sensitized cells and not removed by chloroquine treatment
22	Autoanti-Ce/e	Commercial anti-Jk^b false positive
23	Autoanti-e	False positive with commercial anti-Fy^a and -Jk^b

Conclusion

In this study, no discrepancies were noted when the murine monoclonal antibodies were tested with unsensitized, sensitized, and CDP-treated RBCs (alloantibodies [n=3], autoantibodies [n=20]). The only exception was a potent autoanti-I that caused direct agglutination of all RBCs, as would be expected. Commercial human monoclonal IgM reagents (anti-K, -Jk^a, -Jk^b, and -S) can produce false positive reactions so it is important to test a low-protein diluent control in parallel with the test as suggested by manufacturers to validate the test results. Polyclonal phenotyping antisera derived from a human source are IAT reactive. The RBCs coated with IgG will give a positive result, using the IAT, which accounted for the false positive reactions.

Although previous studies have shown that phenotypes of RBCs sensitized in vivo by alloantibodies can be determined using murine monoclonal antibodies, the nature and characteristics of autoantibodies present in a patient's serum may be quite different.[10] In this paper, we demonstrated that murine monoclonal antibodies may be used without pretreating the RBCs with enzymes or chemicals. Furthermore, they provide accurate, reproducible results. In summary, murine monoclonal antibodies used in this study and in previous studies show that these antibodies can be used to determine the phenotype of patient RBC samples that are DAT positive, thus saving money and time and improving patient care.

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