IgG blocking: An alternative red cell treatment method for phenotyping

SBB Student Research

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ABSTRACT

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Background: Removal of IgG from red blood cells (RBCs) with a positive direct antiglobulin (DAT) is necessary prior to antigen typing with antisera reactive by the antiglobulin test (IAT). The traditional methods using chloroquine diphosphate (CDP) and EDTA glycine acid (EGA) have been successful to obtain DAT-negative RBCs; however, these methods are usually time-consuming or require a complex procedure. Additionally, some RBCs resistant to IgG reduction are occasionally encountered. This study evaluated the effectiveness of a reported simple, rapid, and inexpensive IgG "blocking" (IgG(B) method by performing further parallel testing with CDP and EGA.

Study Design & Methods: A total of 59 RBCs with w+ to 4+ DAT were treated in parallel using CPD, EGA and IgG(B). CDP and EGA treatments were performed following published procedures. For IgG(B), washed packed RBCs were prepared from 30 drops of 3-5% cell suspension; 20 drops of anti-IgG were added and incubated at room temperature (20-26°C) for up to 2 hours or until a negative DAT was obtained; and the RBCs were washed 4 times with isotonic saline and resuspended to a 3-5% cell suspension. Treated RBCs with a negative DAT were typed for the K, S, s, Fy^a, Fy^b, Jk^a and Jk^b antigens. RBCs that did not seroconvert to DAT negative after the initial treatment were retreated up to three total treatments until a negative DAT is obtained.

Results: Of the 59 samples tested, 57 (97%) showed agreement in antigen typing using all three methods. Antigen typing of RBCs treated with IgG(B) showed no significant difference compared to CDP (p=0.34) and EGA (p=0.35). Twelve samples (20%) remained DAT positive after the initial CDP treatment, one (2%) after EGA treatment and six (10%) after IgG(B) treatment. Discrepant K, Fy^a and Jk^a antigen typing results were observed in three of 59 (5%) of samples.

Conclusion: IgG(B) blocks cell-bound IgG based on the ability of DAT-positive RBCs to adsorb anti-IgG. This study confirmed previous finding that IgG(B) compared well with CDP and EGA for IgG reduction and additionally showed that up to three total treatments can be performed on RBCs that did not seroconvert after the initial treatment. IgG(B) can also be used as an adjunct method when CDP or EGA treatment is incomplete.

INTRODUCTION

Red blood cells (RBCs) sensitized with immunoglobulins, as in hemolytic disease of the fetus and newborn, hemolytic transfusion reactions and autoimmune hemolytic anemia, can be expected to have a positive direct antiglobulin test (DAT). Accordingly, IgG-coated RBCs cannot be tested for red cell surface antigens by means of the indirect antiglobulin test (IAT), nor with reagents containing potentiators that have potentials to cause false-positive results.[1] The ability to remove or block immunoglobulin from the cells without impairing surface antigen reactivity is essential for the cells to be antigen typed to aid in the identification of clinically significant alloantibodies.[2,3]

The traditional methods of treating IgG-coated RBCs to render them DAT negative are usually time-consuming or require a complex procedure. These methods include gentle heat elution,[1] chloroquine diphosphate (CDP)[4] and acid glycine/EDTA (EGA).[1] Another method involves adsorbing antisera with RBCs and then test the reactivity of the adsorbed antisera as an indirect method to determine the antigen typing.[5] CDP and/or EGA are usually successful in obtaining DAT-negative RBCs. However, non-seroconversion occasionally occurs in some RBCs with strong positive DAT, indicating the procedural limit has been reached for CDP and/ or EGA.

A quick and simple IgG “blocking” (IgG(B) method for treating IgG-coated red blood cells was previously described.[1] While the mechanism of IgG(B) is not clearly defined, it is believed that cell-bound IgG can be blocked by the addition of anti-IgG. The purpose of this study was to further evaluate the IgG(B) method by expanding the

scope of testing to include more RBC samples, more RBCs with strong DAT (3-4+), and to determine the treatment limit.

MATERIALS AND METHODS

A total of 59 freshly collected anticoagulated blood samples from nontransfused donors with DAT ranging from w+ to 4+ were evaluated. When testing was delayed, the specimens were stored at 1-6 ^{o}C for no longer than 72 hours because RBCs stored longer than 72 hours may show increased hemolysis when treated with blood bank chemicals.

All RBCs were treated in parallel using CDP, EGA and IgG(B). Following treatment, the RBCs were tested for the K, S, s, Fy^a, Fy^b, Jk^a and Jk^b antigens (ImmucorGamma, Inc., Norcross, GA; Ortho Diagnostics, Raritan, NJ). All testing was performed by the tube antiglobulin method and agglutination reactions were scored.[1] DAT and antigen typing were performed using anti-IgG (murine monoclonal green, ImmucorGamma Inc., Norcross, GA). EGA was obtained from a commercial source (ImmucorGamma, Inc., Norcross, GA) and CDP (Sigma Chemicals, St. Louis, MO) was prepared in-house.

Antigen typing was performed in parallel with 6% bovine albumin as a negative control to confirm that the immunoglobulin coating has been successfully removed from the cells. A positive IAT in the negative control invalidates all other testing; these samples were retreated and all testing was repeated.

IgG(B) Modified Method

RBCs to be treated were washed three times with isotonic saline and resuspended to a 3-5% cell suspension. Thirty drops (1.5 mL) of the 3-5% cells were placed into a labeled tube and centrifuged for 1 minute. The supernatant was removed, being careful not to disturb packed red cells. Twenty drops (1 mL) of anti-IgG was added to the packed cells and the mixture incubated at room temperature (20-26°C) for up to 2 hours. The DAT was checked on an aliquot of cells at 30-minute intervals during treatment. RBCs with a weak DAT required shorter treatment time (30-60 minutes) while RBCs with a stronger DAT (>2+) required longer incubation times (1-2 hours) and/or multiple incubation cycles.

After incubation, the antiglobulin-RBC mixture was centrifuged for 30 seconds and the supernatant discarded. The cells were washed 4 times with isotonic saline and resuspended to a 3-5% cell suspension. A DAT was performed. If the DAT was negative, antigen testing was performed according to manufacturer's package inserts. Samples with a positive DAT after the initial treatment were retreated with IgG(B) up to a total of three treatments.

CPD treatment and EGA treatment of RBCs

Blood samples were treated with CDP and EGA following manufacturers' directions. Samples that did not seroconvert to DAT negative after the initial treatment were retreated using IgG(B) up to a total of three treatments until a negative DAT was obtained.

Statistical Analysis

Data were statistically analyzed using paired t-test. The level of significance was established at $p < 0.05$

RESULTS

Of the 59 samples tested, 57 (97%) showed agreement in antigen typing using all three methods (Table 1). Antigen typing of RBCs treated with IgG(B) showed no significant difference compared to CDP ($p=0.34$) and EGA ($p=0.35$) (Table 2).

A total of 12 samples remained DAT positive after the initial treatment: 12 (20%) for CDP, one (2%) for EGA, and six (10%) for IgG(B). Of the 12 samples, three (25%) had 2+ DAT and nine (75%) had 3-4+ DAT (Table 1). One sample with a 4+ DAT (# 45) did not seroconvert to DAT negative using all three methods and following three total treatments.

Discrepant antigen typing results were obtained in three samples. Sample #7 typed K+ and Fy(a+) after IgG(B), but Fy(a-) with EGA and K-, Fy(a-) with CDP. Sample #27 typed Fy(a-) with EGA but Fy(a+) with CDP and IgG(B). Sample #28 typed Jk(a-) with EGA but Jk(a+) with CDP and IgG(B).

Sample #4 (DAT 4+) seroconverted to a DAT negative after a two-time EGA treatment but was reduced to only a 2+ DAT using CDP and/or IgG(B). This sample eventually converted to DAT negative using the three treatment limit with IgG(B) as the second treatment method. However, the test was invalidated because the 6% albumin

control was 2+. Unfortunately, the EGA results could not be used and antigen testing for this sample was omitted.

DISCUSSION

IgG(B) was reported as a quick and simple method for phenotyping IgG-sensitized RBCs.[1] The original report compared IgG(B) with CDP and EGA, testing 26 samples with DAT ranging mostly from w+ to 2+. Of the 26 samples tested, 23 were in agreement by all three methods. One sample with a 2+ DAT typed Fy(b+) using the IgG(B) method, but Fy(b-) with both CDP and EGA. Two samples with 2+ and 4+ DAT respectively, remained DAT positive after the IgG(B) method but were rendered negative with the EGA or CDP method.[1]

This study evaluated a larger sample size of 59 samples and included more samples with strong 4+ positive DAT. Additionally, the treatment limit was evaluated comparing the three methods using a time limit of 2 hours and up to three total treatments. The results of this study were consistent with the previous report that IgG(B) worked well for RBCs with DAT ranging from ± weak to 2+. Results of antigen typing were in agreement by all three methods for 97% samples.

Similar to the original report, discrepant results were noted in this study. Certain samples with weak expression of Fy^a were altered by both CDP and EGA treatment. One sample with discrepant Jk^a typing could also be attributed to weak Jk^a expression, possibly a silent *Jk* allele.[6] The sample with the discrepant K typing may be caused by

technical error. Perhaps with the advancement of molecular testing, the silent *Fy* and *Jk* gene(s) could be elucidated.

The development of molecular techniques has greatly expanded the knowledge of all biologic systems applicable to the diagnosis of disease, the practice of forensic science, the generation of recombinant proteins and the production of functional genes for gene therapy.[1] Advancement in red cell molecular testing has provided an invaluable tool to complement serological testing.[7] However, the use of molecular testing is currently limited by the lack of expertise, time restraints, complexity of typing, unknown genetic variants, expense to patient as well as cost.[8]

In conclusion, this study confirmed previous finding that IgG(B) compared well with CDP and EGA for IgG reduction and additionally showed that up to three total treatments can be performed on RBCs that did not seroconvert after the initial treatment. IgG(B) can also be used as an adjunct method when CDP or EGA treatment is incomplete. RBCs with strong 3-4+ DAT can be first converted to ≤ 2 using either CDP or EGA, followed by IgG(B) to completely seroconvert a negative DAT. Additional studies are needed to determine if a shorter treatment time can be used for IgG(B). The sequence of treatment of using IgG(B) first followed by CDP or EGA can also be evaluated.

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Table 1. Comparison of antigen typing scores using EGA, CDP, and IgG(B) Methods on DAT-positive RBCs

Case #	Treatment DAT IgG				6% alb Ctrl	Antigen typing of treated red blood cells: S			s			K			Fy^a			Fy^b			Jk^a			Jk^b		
	Pre	EGA	CPD	IgG(B)		EGA	CPD	IgG(B)	EGA	CPD	IgG(B)	EGA	CPD	IgG(B)	EGA	CPD	IgG(B)	EGA	CPD	IgG(B)	EGA	CPD	IgG(B)	EGA	CPD	IgG(B)
1	8	0	0	0	0	8	8	8	12	12	12	*	0	0	12	12	12	10	10	10	10	10	10	8	8	8
2	10	0	0	0	0	0	0	0	10	8	10	*	0	0	10	10	10	8	10	12	10	10	10	10	10	10
3	12	0	3	0	0	8	8	12	10	10	10	*	0	0	0	8	0	8	0	8	10	10	10	10	10	10
4	12	3	5	3	8							*														
5	10	0	8	0	0	0	0	0	10	10	12	*	0	0	8	8	10	0	0	0	10	10	10	10	10	10
6	5	0	0	0	0	8	10	8	0	0	0	*	0	0	8	10	10	8	8	5	0	0	0	10	10	10
7	10	0	8	0	0	8	8	8	0	0	0	*	0	8	0	0	8	8	8	8	10	12	10	8	8	8
8	12	0	8	3	0	8	5	10	8	10	10	*	0	0	8	8	8	8	5	8	10	12	10	8	8	8
9	8	0	8	3	0	0	0	0	8	10	10	*	0	0	0	0	0	8	8	8	8	10	10	8	8	8
10	5	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	10	8	8	10	8	10	0	0	0
11	8	0	0	0	0	0	0	0	12	10	12	*	0	0	0	0	0	8	10	8	10	10	10	0	0	0
12	3	0	0	0	0	0	0	0	10	10	10	*	0	0	8	8	8	8	8	8	0	0	0	8	8	8
13	5	0	0	0	0	0	0	0	10	10	10	*	0	0	8	8	8	8	10	10	10	10	10	10	10	10
14	5	0	0	0	0	8	8	8	10	10	10	*	0	0	8	8	10	8	8	8	10	10	10	10	10	10
15	5	0	0	0	0	0	0	0	8	8	8	*	0	0	8	10	10	8	8	8	0	0	0	10	10	10
16	5	0	0	0	0	0	0	0	12	12	12	*	0	0	8	10	10	8	10	10	10	10	10	10	10	10
17	8	0	0	0	0	8	8	8	8	8	10	*	0	0	8	10	12	8	8	10	8	8	10	10	10	10
18	5	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	8	8	8	10	10	10	0	0	0
19	3	0	0	0	0	8	8	10	12	12	12	*	0	0	10	10	10	10	10	10	10	10	10	10	10	10
20	8	0	0	0	0	0	0	0	10	10	10	*	10	10	0	0	0	10	8	8	10	10	10	8	8	8
21	5	0	0	0	0	8	8	8	0	0	0	*	0	0	8	8	8	0	0	0	8	8	8	0	0	0
22	10	0	3	3	0	8	10	10	8	10	10	*	0	0	8	10	10	10	8	8	10	10	10	10	10	10
23	3	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	0	0	0	12	10	12	0	0	0
24	8	0	5	3	0	8	8	10	0	0	0	*	0	0	0	0	0	10	10	8	10	10	10	10	10	10
25	5	0	0	0	0	0	0	0	8	10	10	*	0	0	8	10	8	8	10	10	10	10	10	0	0	0
26	8	0	0	0	0	8	8	8	8	8	8	*	0	0	8	8	10	8	8	8	0	0	0	8	8	8
27	8	0	8	0	0	0	0	0	10	10	10	*	0	0	0	10	10	0	0	0	8	10	10	10	10	10
28	12	0	8	0	0	8	10	8	8	10	10	*	12	8	8	10	10	10	10	8	0	8	10	10	10	10
29	3	0	0	0	0	0	0	0	10	10	10	*	10	10	0	0	0	10	10	10	12	12	12	10	10	10
30	5	0	0	0	0	0	0	0	10	10	10	*	10	10	12	10	10	10	10	10	10	10	10	10	10	10
31	8	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	12	10	10	10	10	10	0	0	0
32	3	0	0	0	0	8	8	8	10	10	10	*	0	0	8	10	10	0	0	0	0	0	0	10	10	10
33	5	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	10	10	10	10	10	10	0	0	0
34	3	0	0	0	0	0	0	0	12	10	10	*	0	0	0	0	0	0	0	0	10	10	10	10	10	10
35	8	0	0	0	0	0	0	0	10	10	10	*	8	8	0	0	0	8	8	8	10	10	10	8	8	8
36	10	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	8	8	8	8	8	8	0	0	0
37	3	0	0	0	0	0	0	0	12	12	12	*	0	0	0	0	0	10	10	10	10	10	10	0	0	0
38	10	0	0	0	0	0	0	0	12	10	12	*	0	0	10	10	10	0	0	0	8	8	8	0	0	0
39	10	0	10	0	0	10	10	8	10	10	10	*	0	0	10	10	10	0	0	0	0	0	0	10	10	10
40	8	0	0	0	0	0	0	0	10	10	10	*	0	0	10	10	10	10	10	8	12	12	12	10	10	10
41	3	0	0	0	0	10	10	10	12	10	12	*	0	0	0	0	0	10	10	10	10	10	10	0	0	0
42	3	0	0	0	0	10	8	10	10	10	10	*	0	0	8	8	8	8	8	10	0	0	0	10	10	10
43	8	0	0	0	0	0	0	0	10	10	10	*	0	0	8	8	8	8	8	10	0	0	0	10	10	10
44	10	0	0	0	0	0	0	0	12	10	10	*	0	0	10	10	10	0	0	0	10	10	10	0	0	0
45	12	8	12	12	0							*														
46	5	0	0	0	0	0	0	0	10	12	12	*	0	0	0	0	0	0	0	0	10	10	10	10	10	10
47	10	0	0	0	0	0	0	0	10	10	10	*	0	0	10	8	8	0	0	0	8	8	8	0	0	0
48	8	0	0	0	0	0	0	0	10	8	10	*	0	0	0	0	0	10	10	10	10	10	10	0	0	0
49	3	0	0	0	0	0	0	0	10	10	10	*	0	0	10	10	10	0	0	0	0	0	0	10	10	10
50	3	0	0	0	0	0	0	0	8	10	10	*	10	10	8	8	8	8	8	8	0	0	0	10	10	10
51	5	0	0	0	0	0	0	0	8	10	8	*	0	0	0	0	0	0	0	0	0	0	0	8	8	8
52	8	0	0	0	0	0	0	0	8	10	10	*	0	0	8	8	8	0	0	0	0	0	0	8	8	10
53	5	0	0	0	0	0	0	0	8	10	10	*	0	0	8	8	8	0	0	0	0	0	0	8	8	8
54	10	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	8	10	5	10	10	10	0	0	0
55	3	0	0	0	0	0	0	0	10	10	10	*	0	0	8	10	8	10	10	10	10	10	10	10	10	10
56	3	0	0	0	0	0	0	0	10	10	10	*	0	0	0	0	0	8	8	10	10	10	10	0	0	0
57	3	0	0	0	0	0	0	0	10	10	10	*	0	0	8	8	10	8	8	10	10	10	10	0	0	0
58	3	0	0	0	0	0	0	0	10	10	10	*	0	0	8	8	8	0	0	0	0	0	0	10	10	10
59	3	0	0	0	0	0	0	0	10	10	12	*	10	8	8	8	8	8	8	8	8	8	8	8	8	8

*Not Tested
Scoring: 4+ =12, 3+ =10, 2+ =8, 1+ =5

Table 2. Comparison of the effectiveness of IgG(B) with CDP and EGA

p value	CDP vs IgG(B)	EGA vs IgG(B)
Anti-S	0.13	0.05
Anti-s	0.16	0.15
Anti-K	0.41	NA
Anti-Fy^a	0.31	0.01
Anti-Fy^b	0.28	1.67
Anti-Jk^a	0.21	0.07
Anti-Jk^b	0.16	0.16
Mean p-value	0.34	0.35