Use of a recombinant deacetylase to convert A₁ red blood cells to the acquired B phenotype for quality control purposes

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INTRODUCTION

A full ABO blood grouping test involves determining the ABO antigens on the red blood cells (RBCs) as well as identifying the corresponding antibodies in the plasma according to Landsteiner's rule. This is achieved using validated and highly regulated anti-sera as well as specific donor test RBCs. However, interpretation of typing results can be complicated by both inherited and acquired factors. Genetic variants weakening the expression of A and B antigens are caught by routine typing but acquired phenotypes may easily go undetected. The acquired B (acqB) phenomenon was first described in 1959 by Cameron et al. who observed a weakly reactive B antigen in samples from seven unrelated patients previously typed as group A¹. Family studies excluded the presence of an inherited weak B antigen. Serum contained anti-B in all cases and only A and H antigens were detected in saliva from the secretors. Subsequent cases indicated that the phenomenon appeared to be associated with age or disease since all were elderly, and all had carcinoma of the rectum and/or colon.² Elegant work by Gerbal et al.³ showed that bacterial enzymes can cause conversion of the immunodominant blood group A sugar, N-acetylgalactosamine, into a structure that highly resembles the corresponding blood group B sugar structure, galactose. This antigen modification ceases following clearing of the underlying bacterial infection. Although the acqB phenotype is rare and transient, there is a risk for errors in blood grouping as shown by a case of fatal hemolytic transfusion reaction in an elderly patient who developed the acqB phenotype and was transfused with four units of group AB erythrocytes following compatible immediate spin cross matching.⁴ Prior to transfusion the patient was mistyped as group AB due to the use of monoclonal anti-B sera (clone ES-4) in routine use at the time. Subsequent investigation into the patient's RBCs with other monoclonal anti-B sera and several polyclonal anti-B did not detect the acqB phenotype. The patient's serum was later shown to contain a weakly reactive anti-B, only detected by the indirect antiglobulin (IAT) test. As a result, the ES-4 clone was withdrawn pending further investigation. This is not a unique case as Pedreira and Noto observed 13 discrepancies out of 25,111 samples during a nine month period after the introduction of monoclonal anti-B for routine ABO typing.⁵ All were attributed to the ES-4 clone, and only two reacted with polyclonal anti-B. Regulatory agencies recommend that any new anti-B reagent is formulated to be non-reactive with acqB cells, to avoid mistyping. However, this has created a Catch-22 situation since we no longer detect acqB with current monoclonal anti-B reagents. Therefore, these RBCs are not readily available to blood typing reagent manufacturers for product quality control, nor to most reference laboratories when anti-B reagents are tested and compared prior to use in clinical practice. Recently, Rahfeld et al.
described a novel approach for enzymatic conversion of group A RBCs to group O using an enzyme pair from the obligate anaerobe \textit{Flavonifractor plautii} that work in concert to efficiently convert the A antigen to the H antigen\(^{6}\). In this two-step enzymatic digestion, the terminal group A sugar, N-acetylgalactosamine, is first deacetylated to a galactosamine intermediate using the enzyme, \(\text{FpGalNAcDeAc}\) and then removed by \(\text{FpGalactosaminidase (FpGalNase)}\)\(^{6}\). We recognised that the initial deacetylation step could be effectively used to produce reagent acqB cells for quality control purposes (Figure 1a).

**MATERIALS AND METHODS**

**Blood Samples**

Anonymized, acid citrate dextrose (ACD)-anticoagulated blood samples, collected as part of the routine blood donation procedure, were obtained from the Department of Clinical Immunology and Transfusion Medicine, Office for Medical Services, Region Skåne, Sweden, following approval (2018:22 and 2020:16) in accordance with the Swedish research law.

**Enzyme treatment**

\(\text{FpGalNAcDeAc}\) was produced as per the protocols previously published\(^{6}\). Aliquots of group A, B and O RBCs were washed three times with phosphate-buffered saline (PBS; Gibco, Lot 1930728 [Thermo Fisher Scientific, Waltham, MA USA]), pH 7.4. Packed RBCs were diluted to 50% hematocrit with PBS and treated with \(\text{FpGalNAcDeAc}\) at two concentrations: 50 µg/mL and 250 µg/mL for 1 hour at 3\(^{\circ}\)C (with gentle mixing every 15 min). Thereafter, RBCs were washed three times with PBS. Thereafter, 1 and 3% solutions in a low ionic preservative solution (ID-Cellstab; lot. 057040012, BioRad [Hercules, CA, USA]) were prepared for testing.

**Figure 1** - Schematic representation of the structure and reactivity of terminal trisaccharides

(a) \(\text{FpGalNAcDeAc}\) treatment removes the acetyl group from the terminal - N-acetylgalactosamine of blood group antigen A, resulting in the acquired B structure, galactosamine. Blood group B antigen shown as comparison. Functional groups highlighted by red arrows. Modified from Rahfeld \textit{et al.}\(^{6}\). (b) \(\text{FpGalNAcDeAc}\)-treated A\(_1\) RBC reacted 3+ and 1+ with anti-B from clone ES-4 (Immucor and Gamma), but not with monoclonal anti-A or monoclonal anti-B (Novaclone ref. 5175). Untreated A\(_1\) control RBCs reacted 4+ as expected with monoclonal anti-A, but not with anti-B (including clone ES-4).
Serological testing
Standard serological techniques were used to evaluate the enzymatic modification of RBCs. Treated and untreated RBCs were tested by a panel of ABO reagents (Table I) in neutral gel cards (ID: 50520, NACL, Enzyme test and cold agglutinins, BioRad) and in tubes according to the manufacturer’s recommendations. In brief, for gel cards 50 µL 1% RBCs + 25 µL antisera were incubated for 10 min at room temperature (RT) followed by centrifugation. 3% RBCs were tested by an immediate spin test in tubes, 1 drop RBCs + 1 drop antisera. Additionally, the RBCs were tested in the following ABO typing cards: DiaClon ABO/D + Reverse Grouping (ID: 50741, Bio-Rad) and ABD-Confirmation for Donors (ID: 51051, Bio-Rad).

Glycerol preservation and recovery
Enzyme-treated RBCs were frozen according to the glycerol preservation and recovery protocol from Judd’s Methods in Immunohematology modified as follows: equal volumes RBCs and glycerol (57%, S.A.L.F. S.p.A. Laboratorio Farmacologico, Cenate Sotto, Italy) were separately warmed to RT. Glycerol (¼ of the total volume) was added dropwise to the RBCs under gentle agitation. The RBCs were equilibrated at RT for at least 3 min before adding the rest of the glycerol, without agitation. The solution was mixed well before dispersing into cryotubes, frozen and stored at −80°C. RBCs were recovered as follows: 0.6 volumes of 12% NaCl (in a 10-mL tube) to 1 volume RBCs was preheated at 37°C in a water bath. RBCs were thawed at 37°C and transferred dropwise to the NaCl under gentle agitation. The solution was equilibrated for 5 min at RT before slowly adding PBS. The tube was centrifuged for 5 min at a low setting (600 x g) and washed continually with PBS until the supernatant was clear. Thawed RBCs were diluted in ID-CellStab, tested with monoclonal anti-A, anti-B as well as the ES-4 clone as described above and subsequently stored at 4°C.

RESULTS
Enzymatically modified A RBCs reacted 3+ and 1+ with the ES-4 clone from two different manufacturers, but not with another monoclonal anti-B blend (B84+B97) (Figure 1b). Loss of reactivity with anti-A was also noted after treatment. Currently, four monoclonal anti-B are in use at our reference lab, two are used for rapid typing (tube) and two are in routine ABO routine typing (gel column agglutination). None detected the acquired B antigen. To increase our reagent pool, we included one additional anti-A and two anti-B reagents, used for research purposes (Table I) showing identical results. Group B RBCs retained reactivity with anti-B upon enzyme treatment and group O RBCs remained non-reactive with both anti-A and anti-B reagents. Hence, specific deacetylation of the terminal N-acetylgalactosamine on A antigen on the A1 RBCs was achieved. The acquired B phenotype remained stable upon freezing, thawing and subsequent storage in ID-Cellstab, up to a period of four months at 4°C. Crossmatch tests showed 1 to 3+ reactions in 17/26 group A plasmas (Table II), but not with their untreated controls (not shown). Anti-A reactivity in group B plasmas

Table I - Anti-A and anti-B reagents used in this study

<table>
<thead>
<tr>
<th></th>
<th>Clone</th>
<th>Use</th>
<th>Reference</th>
<th>Manufacturer</th>
</tr>
</thead>
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<tr>
<td>Anti-A</td>
<td>Birma 1</td>
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<td>lot.101030E</td>
<td>Immucor/Immucor</td>
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<tr>
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<td>9113D10</td>
<td>research only</td>
<td>lot.300000</td>
<td>Diagast</td>
</tr>
<tr>
<td>Anti-B</td>
<td>B84/B97</td>
<td>quick spin blood typing (tube test)</td>
<td>5175/lot.204199</td>
<td>Novaclone/Immucor</td>
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<tr>
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<td>G1/2</td>
<td>quick spin blood typing (tube test)</td>
<td>10301</td>
<td>Diaclone</td>
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<tr>
<td></td>
<td>ES-4</td>
<td>research only</td>
<td>A46253</td>
<td>Immucor</td>
</tr>
<tr>
<td></td>
<td>ES-4</td>
<td>research only</td>
<td>lot.BM36-3</td>
<td>Gamma/Immucor</td>
</tr>
<tr>
<td>DiaClon ABO/D (DVI-, DVI-) + Reverse grouping</td>
<td>Anti-A: A5 Anti-B: G1/2</td>
<td>routine blood typing (gel column agglutination)</td>
<td>ID: 50741</td>
<td>Bio-Rad</td>
</tr>
</tbody>
</table>

Immucor, Norcross, GA, USA; Diagast, Loos, France; Diaclone, Besançon, France; BioRad, Hercules, CA, USA.

Table II - Crossmatch results: FpGalNAcDeAc-treated A1 RBCs tested with a panel of donor plasma

<table>
<thead>
<tr>
<th>Donor plasma panel</th>
<th>Group</th>
<th>4+</th>
<th>3+</th>
<th>2+</th>
<th>1+</th>
<th>Negative</th>
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<tr>
<td>A (No.=26)</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>B (No.=6)</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AB (No.=3)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
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</table>

Crossmatch reactions are graded 4+, 3+, 2+, 1+ and negative.

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was completely abolished with 4/6 acqB samples, where 2 samples reacted only weakly. Interestingly, 2/3 group AB plasmas reacted strongly with the modified RBCs.

DISCUSSION
Reagent manufacturers are urged by the Food and Drug Administration and similar agencies world-wide to test their anti-A and anti-B with RBCs expressing common variants of A and B antigen. E.g. this includes testing anti-A with RBCs of the A\textsubscript{1} subgroup. As shown by Pedreira and Noto, the acqB phenotype is more frequent than might be expected among hospital patients, with an incidence in their study of approximately 1 in 850 group A individuals\textsuperscript{5}. Thus, it is important that falsely positive blood grouping reactions are avoided. However, patients’ acqB RBCs do not make good quality control reagents and are now difficult to detect and obtain. Consequently, new antisera risk going untested. Frame et al. describe a method using Kode\textsuperscript{\textregistered} Technology, inserting functional spacer lipid molecules carrying the terminal trisaccharide acqB epitope, and successfully dissecting a panel of monoclonal anti-B including the ES-4 clone\textsuperscript{9,10}. We instead describe an alternative strategy of producing RBCs with the acqB phenotype by treating native group A, RBCs with FpGalNacDeAc. The digestion with FpGalNacDeAc is specific and the modified RBCs remain stable over a period of four months in a RBC preservative solution, or alternatively as frozen and thawed acqB aliquots. Furthermore, our easily reproducible and cost-effective alternative mimics the way acquired B is formed in vivo and does not involve any artificial glycan constructs. Monoclonal anti-B reagents typically do not react with acqB, and a subsequent investigation showed that acidifying ES-4 to pH6.0 eliminated this unwanted reactivity. However, acidification of reagents has been shown to cause false positive reactions due to polyagglutination\textsuperscript{11}.

Interestingly, we also found that 65% of group A plasma contained an antibody that reacted with acqB RBCs. While we cannot exclude that this represents a more broadly reactive anti-B in these plasmas, the variation in antigen strength contradicts this possibility in part as does the strong reactivity of 2 of 3 group AB plasma samples with acqB RBCs, suggesting that this is a specific anti-acqB. While we have specifically exposed the acqB epitope on these RBCs, the results highlight the complexity of the human polyclonal response to carbohydrate antigens and brings to mind previous studies performed on enzyme-converted group O RBCs, namely early work by Kruskall et al.\textsuperscript{14}, that showed unexpected crossmatch reactivity with enzymatically converted group O RBCs, and a more recent report by Moon et al.\textsuperscript{3} suggesting that there are other immunogenic carbohydrate epitopes of potential clinical importance. We have also shown the existence of specific antibodies to extended ABO structures, notably ExtB, again indicating the complexity of human sera to ABO-related antigens\textsuperscript{14}.

CONCLUSIONS
Specific deacetylation of the A antigen by recombinant enzymes can be used to prepare acqB RBCs for quality control of blood grouping reagents and thus add an extra level of safety to ABO blood grouping.

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AUTHORSHIP CONTRIBUTIONS
JRH, JRS and PR designed the study; JRH and JRS performed experiments and JRS, JRH and PR analyzed the data. JRH and JRS drafted the manuscript. All Authors contributed to the overall review of data, edited, and approved the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST
JRH, MLO and JRS have no conflicts of interest relating to the data presented in this paper however, MLO and JRS are joint owners of a consulting firm, BLUsang AB, into which royalties on patents applicable to blood group genotyping, as well as honoraria for speaking engagements and editorial work are paid. SGW, JK and PR are inventors on a patent covering the enzymes employed and co-founders of a company (Avivo Biomedical Inc.) that has licensed that patent.

REFERENCES

Blood Transfus 2023; doi: 10.2450/BloodTransfus.584
Making acquired B test cells


