Problems highlighted when using anticoagulated samples in the standard tube low ionic strength antiglobulin test

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Within the UK blood transfusion services, there is currently no recommendation for the use of either clotted or anticoagulated samples for antibody identification testing. This report describes three cases in which the detection of IgM antibodies was impeded by the use of anticoagulated samples. Two patient samples, referred for compatibility testing, were both identified as having IgM complement-activating anti-S and the remaining case involved an antenatal patient with IgM complement-activating anti-Vel. In all three cases, the coincidental referral and investigation of both clotted and anticoagulated samples led to the discrepancy in serum and plasma test results becoming apparent. Potential errors in selection of suitable blood for transfusion and appropriate antenatal management were avoided by correct identification of the antibodies present using the clotted samples. Immunohematology 2006;22:72–77.

Key Words: antibody detection, plasma or serum, anti-S, anti-Vel, antiglobulin test

The suitability of anticoagulated or clotted samples for pretransfusion testing has been the object of investigation. Kidd antibodies, in particular, have received close scrutiny due to their association with delayed hemolytic transfusion reactions, their ability to activate complement, and the earlier reported difficulty of their detection in systems with reduced sensitivity to complement-activating antibodies. It is widely accepted that, given the improvements in antibody detection by the antiglobulin test (AGT) resulting from the use of different enhancement media (e.g., LISS, polybrene, and PEG) and the advances in AGT technology (gel and bead techniques), the necessity to detect complement activation by capable IgG antibodies has been reduced and possibly eliminated. The general conclusion is there is no significant difference in detection rates when using anticoagulated or clotted samples.

The Welsh Blood Service (WBS) laboratories provide an antenatal antibody screening service for seven hospitals within the region as well as a reference service for serologic investigations for 15 hospitals. The majority of hospital blood banks served use automated sampling systems for RBC typing and antibody investigations and, consequently, use anticoagulated samples.

Subsequently, anticoagulated samples were increasingly referred to the WBS for routine antenatal and patient antibody investigations. Reported here are three cases that serve to emphasize the limitations of anticoagulated samples used by the current testing protocol of the WBS when performing RBC antibody investigations. In each case, the detection of an IgM complement-activating antibody was impeded by the use of anticoagulated samples.

Materials and Methods

Routine tests for antibody identification were performed; they included the standard tube AGT using 1.5% LISS-suspended RBCs incubated at 37°C for 30 minutes, an agglutination test using PBS-suspended RBCs incubated at 18°C for 60 minutes, and a two-stage prepaainized agglutination test incubated at 37°C for 30 minutes. DiaMed column gel ID AGT cards (DiaMed AG, Cressier sur Morat, Switzerland) were used according to manufacturer's instructions. Polyspecific anti-human globulin (AHG) reagent (i.e., combination of anti-IgG and anti-C3d) was used for both the LISS tube test (Lorne Laboratories, Reading, UK) and DiaMed ID cards (DiaMed AG). All tests were performed using plasma and serum samples with appropriate nine-cell panels.
Variation in the pattern of reactivity obtained when testing plasma and serum samples with the panel RBCs by the AGT implied that the detected antibody was a complement-dependent IgM antibody. A series of further tests was performed to confirm the presence of a complement-dependent IgM antibody. Treatment of the serum samples with dithiothreitol (DTT) (Sigma-Aldrich, Steinheim, Germany) was performed to verify the presence of an IgM antibody. Equal volumes of 0.01 M DTT were incubated with the patient’s serum for 30 minutes at room temperature. PBS was added to the patient’s serum in a second tube and similarly incubated as a control. Doubling dilutions were performed on the patient’s untreated serum, DTT-treated serum, and PBS control serum. All dilutions were tested using appropriate RBCs by a LISS-AGT. Reactions were graded macroscopically; due to variation in interpretation of reaction grades, a twofold difference in titer result was considered to be significant.

A two-stage EDTA test was also performed on those anticoagulated patient samples in which a complement-dependent antibody was suspected and when the anticoagulated sample did not react by the tube LISS AGT. A neutralized EDTA solution at pH 7.2 was prepared using 4.0 g of K$_2$EDTA and 0.3 g of NaOH in 100 mL of distilled water. EDTA solution was added to the patient’s plasma sample at a 1 in 10 dilution with incubation at room temperature for 10 minutes. Two volumes of the treated plasma were subsequently incubated with reagent panel RBCs suspended in PBS for 60 minutes at 37°C. After incubation, the RBCs were washed and incubated for 15 minutes with a fresh source of complement. (The complement was derived from a pool of group-compatible non-transfused donor sera that tested negative for the presence of irregular RBC antibodies and was stored frozen within 24 hours of donation.) After additional washing, polyspecific AHG reagent was added and the test was centrifuged and read.

The clinical significance of RBC IgG antibodies can be investigated using a chemiluminescence (CL) test based on the method described by Downing et al.\textsuperscript{5} In the following cases, where appropriate, the CL assay was performed to demonstrate the absence of a clinically significant IgG antibody. Briefly, the patient’s serum was incubated separately with antigen-positive and antigen-negative RBCs. After washing, the sensitized RBCs were incubated with the mononuclear cell preparation in the presence of luminol. Monocyte activation, as indicated by increased chemiluminescence, was measured every 4 minutes for 2 hours. The CL test results were reported as an opsonic index; an opsonic index < 1.2 is indicative of a clinically insignificant IgG antibody.

In each case, compatibility testing was required and performed using serum samples tested with RBCs antigen-negative for the previously identified antibody by a LISS AGT.

Case Reports

Case 1

In 1991, a 50-year-old woman was admitted to a local hospital with a Hb of 8.3 g/dL after a viral infection. Serologic investigations revealed the presence of anti-D and a strongly positive DAT. The patient was diagnosed with autoimmune hemolytic anemia. Transfusion was required on three occasions from 1991 to 1999; the patient received a total of nine units of RBCs. In 1999, a second antibody was detected in the patient’s serum and samples were referred to the WBS for antibody identification and compatibility testing. The presence of anti-S and anti-D was confirmed. In January 2002, the patient presented with a Hb of 7.8 g/dL and was referred for compatibility testing presplenectomy. Pretransfusion testing was performed using anticoagulated samples. The anti-S was no longer detectable by the LISS AGT; however anti-S could be detected by a saline 18°C tube agglutination test. Additional anticoagulated and clotted samples were requested to further investigate the initial findings.

The patient’s RBCs were grouped as A, D– (rr) and were found to react in the DAT with polyspecific and monospecific anti-C3 AHG reagents; negative reactions were obtained when the RBCs were tested using anti-IgG AHG. Routine antibody identification panels using plasma revealed the presence of anti-D reactive by LISS AGT; the anti-D was not confirmed active by papain tests due to the presence of a nonspecific papain autoantibody. Anti-S was detectable by saline 18°C agglutination tube tests and in the LISS AGT before washing. The anti-S was not detectable after washing, addition of AHG reagent, and centrifugation. The anti-S was also detectable when performing a DiaMed gel card AGT. We suspected the presence of an IgM complement-activating anti-S and we repeated our investigations using serum samples (Table 1).

Antiglobulin test titrations were performed using D+ ss, D– Ss, and D– rr ss LISS-suspended RBCs.
Titration of the patient’s serum demonstrated the anti-D to be detectable at a 1 in 16 dilution and anti-S at a 1 in 4 dilution. DTT treatment of the patient’s serum destroyed anti-S activity such that no reactions were observed in the titrations by the AGT; the anti-D remained detectable at a dilution of 1 in 8. As expected, no reactions were obtained with D– rr ss RBCs (Table 2).

Further testing was performed to confirm the characteristics of the antibody (Table 3). The anti-S was detectable by a normal ionic strength saline (NISS) AGT, therefore excluding the possibility that the detection of the anti-S was a LISS phenomenon as had been previously described with other antibody specificities. The use of monospecific anti-IgG rendered the anti-S undetectable in the LISS AGT even with the use of a serum sample. Our results confirmed the anti-S to be an IgM complement-activating antibody. Failure of the patient’s RBCs to react with the anti-S detected by saline 18°C agglutination tests suggested the anti-S to be an alloantibody; this was confirmed by determining the patient’s RBCs to be S– using an in-house IgG anti-S typing reagent.

The patient was transfused with six units of group A, D– (rr) ss RBCs units without incident; postoperative Hb was 13.2 g/dL.

Case 2

In December 2001, a sample was received from an antenatal patient at 11 weeks’ gestation for routine antenatal screening. The patient had two previous pregnancies with no record of any previous transfusions and no irregular antibodies detected during previous antenatal screening. Clotted and anticoagulated samples were received for antenatal antibody screening. Automated testing was performed using the anticoagulated sample with bromelinized RBCs on the Olympus PK7200 (Olympus, Tokyo, Japan) by hemagglutination and using the clotted sample by a solid phase antiglobulin test (SPAT) on the Tecan Genesis RSP 150 (Tecan Schweiz AG, Männedorf, Switzerland). The sample was identified as antibody positive by the SPAT method on the Tecan Genesis RSP 150. The sample was then referred to the Patient Diagnostic Services department for antibody identification and titration as necessary.

Routine antibody identification panels using the patient’s serum sample revealed the presence of an antibody that reacted with all panel RBCs tested by papain and LISS AGT with a negative autocontrol. The patient’s serum failed to react with the Vel– RBC. Anti-Vel was confirmed by testing with additional examples of Vel– RBCs. No additional RBC antibodies were detected and the patient’s own RBCs typed as Vel–. Due to insufficient serum, further testing by the AGT was performed using the referred anticoagulated sample. Before the AGT washing phase, agglutination was observed throughout the LISS panel when using the anticoagulated sample; however, after washing, the anti-Vel was undetectable by LISS AGT (Table 4).

Vel antibodies are characteristically IgM complement-activating antibodies; therefore, predictably discrepant results were obtained when

Table 1. Case 1: results of routine antibody identification panels

<table>
<thead>
<tr>
<th>Saline 18°C test</th>
<th>Preparainapized test</th>
<th>LISS AGT</th>
<th>DiaMed test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sample</td>
<td>Anti-S PNSA*</td>
<td>Anti-D†</td>
<td>Anti-D and anti-S</td>
</tr>
<tr>
<td>Serum sample</td>
<td>Anti-S PNSA*</td>
<td>Anti-D and anti-S</td>
<td>Anti-D and anti-S</td>
</tr>
</tbody>
</table>

*An apparently nonspecific papain autoantibody detected.
†Before AGT washing, positive reactions giving anti-S pattern were observed.

Table 2. Case 1: results of AGT titrations using untreated and DTT-treated serum

<table>
<thead>
<tr>
<th>RBC phenotype</th>
<th>Neat</th>
<th>1:2 Dilution</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated serum sample</td>
<td>R, R, ss</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2†</td>
</tr>
<tr>
<td></td>
<td>rr ss</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DTT-treated serum sample</td>
<td>R, R, ss</td>
<td>NA</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>rr ss</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PBS control serum sample</td>
<td>R, R, ss</td>
<td>NA</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rr ss</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Reactions graded according to system described in Table 12.2 Guidelines for Blood Transfusion Services in the United Kingdom.
†Endpoints of titration in bold figures.

Table 3. Case 1: results of additional testing by AGT

<table>
<thead>
<tr>
<th>LISS AGT using polyspecific AHG</th>
<th>LISS AGT using anti-IgG AHG</th>
<th>NISS AGT using polyspecific AHG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum sample</td>
<td>Anti-D and anti-S</td>
<td>Anti-D*</td>
</tr>
<tr>
<td>Plasma sample</td>
<td>Anti-D*</td>
<td>Anti-D*</td>
</tr>
</tbody>
</table>

*Before AGT washing, positive reactions demonstrating anti-S pattern were observed.
testing plasma and serum samples. The plasma sample was further tested by a two-stage EDTA AGT. This technique permits the detection of complement-activating antibodies in plasma samples by the subsequent incubation of IgM-sensitized RBCs with a fresh source of complement. Complement enhancement of the routine tests by a two-stage EDTA AGT revealed the anti-Vel.

Titration of the patient’s serum demonstrated anti-Vel detectable at a 1 in 4 dilution. DTT treatment of the patient’s serum (as previously described) destroyed the anti-Vel activity such that no reactions were observed in the titrations by the AGT (Table 5).

IgM Vel antibodies are not associated with HDN. The clinical significance of anti-Vel with respect to its ability to cause HDN was confirmed using a CL test. CL assay results were negative (opsionic index < 1.2). Results of the serologic investigations confirmed the anti-Vel as an IgM antibody; the CL test results were, therefore, as expected.

Continuous monitoring of the antibody throughout pregnancy was undertaken and samples from the patient were referred on five additional occasions. On each occasion, the antibody specificity and class were confirmed and additional specificities were excluded. Four units of group-compatible Vel– RBCs were kept on standby for the mother before the expected date of delivery. The patient delivered at 40 weeks’ gestation, without RBC transfusion support. The cord Hb was 21.9 g/dL, bilirubin was 61 µmol/L rising to 90 µmol/L, and the DAT was negative; all parameters indicated no HDN.

Case 3

In November 2003, a 70-year-old man was referred for preoperative investigations and compatibility testing. Anticoagulated and clotted samples were referred and initial investigations were performed on the anticoagulated samples because the clotted samples were inappropriately labeled. No reactions were detected by LISS AGT using the anticoagulated samples; however, a pattern of reactivity was detected by a saline 18°C agglutination test. Further testing was performed comparing the reactivity of the serum and plasma samples.

Initial antibody investigation results using anticoagulated samples suggested the presence of an IgM antibody detected by the saline 18°C agglutination test. The presence of a weak anti-S was confirmed by tube LISS AGT using clotted samples and a DiaMed (DiaMed AG) gel test using both anticoagulated and clotted samples. The anti-S demonstrated typical dosage effects and did not react with all heterozygous Ss RBCs. Titrations using the patient’s untreated, DTT-treated, and PBS control sera were inconclusive due to the lack of reactivity with diluted serum. The patient’s serum did not react with SS panel RBCs when tested by a NISS AGT, indicating that the antibody is LISS dependent.

The patient was successfully transfused with five units of ABO and Rh phenotype compatible, ss RBCs with a posttransfusion Hb of 12.2 g/dL.

Discussion

All three cases demonstrate the failings in the tube LISS AGT to detect complement-activating IgM antibodies when using anticoagulated samples. Coincidental testing of plasma and serum samples demonstrated the differences in antibody activity but it poses the question of how many complement-activating IgM antibodies remain undetected in plasma samples. The clinical significance of the IgM anti-S identified in cases 1 and 3 is unknown. S antibodies of IgM class are rarely reported and both patients reported here received S- RBCs. Anti-S can cause immediate and delayed hemolytic transfusion reactions and IgG anti-S has been implicated in HDN. It is
therefore necessary that anti-S be detected during pretransfusion testing.

Anti-Vel is rarely associated with cases of HDN; the Vel antigen is not fully developed at birth and examples of IgG anti-Vel are rare. Confirmation of the lack of clinical significance of the anti-Vel in relation to HDN was provided by a negative CL test result. Antenatal screening, however, not only allows for the identification of antibodies that may cause problems to the fetus and neonate but also alerts the blood bank of the presence of antibodies that may cause pretransfusion testing problems. Anti-Vel has been implicated in hemolytic transfusion reactions and failure to identify this antibody could result in a hemolytic transfusion reaction if Vel+ RBCs were transfused. In addition, the frequency of Vel- RBCs is 1 in 3711; failure to identify this antibody before delivery could have resulted in significant delay in the provision of compatible blood, if required.

The British Standards and Controls in Haematology Guidelines for pretransfusion compatibility procedures in blood transfusion laboratories do not advocate the use of serum over anticoagulated samples, but they recommend validation of any changes to the sample used for testing. Serum samples, however, were recommended for the investigation of suspected hemolytic transfusion reactions in the annual Serious Hazards of Transfusion report (2001-2002) in recognition of the ability to detect weak complement-binding antibodies in serum samples. Studies investigating the suitability of samples for pretransfusion testing conclude that there are no significant differences in detection rates when using either plasma or serum. There is no single technique that will consistently outperform all other techniques in the detection of clinically significant RBC antibodies, but each laboratory must be aware of any limitations of the test they perform. Because the WBS is a reference center for RBC serology referrals, room temperature agglutination tests are performed to assist in the identification of cold-reactive antibodies in complex antibody mixtures that may interfere with LISS AGT. Failure to have performed saline 18°C agglutination tests or to have noted the pattern of reactions before washing could have resulted in the antibodies in these case studies remaining undetected in the anticoagulated samples. In each case, the use of a clotted sample tested by both automated and manual techniques performed at the WBS, as well as the application of good serologic practices, facilitated the detection of these antibodies. The standard DiaMed gel AGT was performed in both cases where anti-S was identified. Contrary to the results of the tube LISS AGT, anti-S was detectable using both serum and plasma samples by the DiaMed AGT. Although there is much debate as to the sensitivity of the DiaMed test when compared with the traditional LISS tube AGT, DiaMed gel tests proved more sensitive for the detection of IgM antibodies when using plasma samples in the cases discussed. The washing phase in the LISS tube AGT disrupts IgM agglutinates and, consequently, the detection of both the anti-S and anti-Vel antibodies using polyspecific AHG relies entirely on the activation of complement. Lack of complement activation when using plasma samples therefore inhibits the detection of these antibodies by the LISS tube AGT. The omission of a washing phase when using DiaMed AGT cards allows IgM agglutinates to remain intact. Therefore, the anti-S and anti-Vel antibodies were detectable in plasma samples when using the DiaMed AGT cards despite the lack of complement activation.

These cases are of interest because of the rare occurrence of anti-S of IgM class and of anti-Vel. They also serve to highlight the benefit of using clotted samples, particularly when performing tube tests. The WBS commenced routine antenatal screening by the DiaMed technique using anticoagulated samples in November 2004 and continues to use the standard tube LISS AGT for the reference work performed. We request the referral of clotted samples for all reference work.

Acknowledgments
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References
Plasma or serum for red cell serology?


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