Sulfhydryl treatment of serum or plasma for the reduction of IgM antibodies

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Dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) are sulfhydryl compounds that can be used to treat serum or plasma to denature IgM antibody reactivity. By using sulfhydryl agents, IgG and IgM antibodies can be separated, the relative amount of IgM and IgG antibodies can be determined, and the risk of hemolytic disease of the fetus and newborn can be assessed. ***Immunohematology* 2018;34:135–139.**

Key Words: dithiothreitol, 2-mercaptoethanol, IgM antibodies

Principle

An IgM molecule is a 19S pentamer in which disulfide bonds hold the five immunoglobulin subunits together; a J chain joins them. These intersubunit disulfide bonds and J chain are more sensitive to reduction with sulfhydryl reagents than the interchain (heavy and light chains) disulfide bonds that are in both IgM and IgG molecules. Dissociating the IgM pentamer into its single subunits helps to identify the immunoglobulin class of an antibody and determine its potential for causing hemolytic disease of the fetus and newborn (HDFN).[1]

Sulfhydryl groups, or thiols, such as dithiothreitol (DTT) and 2-mercaptoethanol (2-ME), are carbon-based molecules bound to a sulfur and hydrogen atom (R-SH). These thiols can reduce disulfide bonds (R-SS-R) in proteins and can be oxidized to form disulfide bonds (R-SH + R-SS-R ↔ R-SS-R + R-SH).[2]

Reactivity of IgM antibodies can be removed through the dissociation of the intersubunit disulfide bonds and J chains (Fig. 1). Reactivity of IgG antibodies may be enhanced through the reduction of disulfide bonds in the hinge region, allowing for binding of antigen sites further apart.[1]

In 1957, Deutsch and Morton[2] described the use of 2-mercaptoethanol (2-ME) to dissociate human macroglobulins, which reaggregated into different molecular components after dialysis to remove the sulfhydryl compound. The use of sulfhydryl-blocking agents prevented the reaggregation.[2]

Reagents/Supplies

Reagents	Supplies
▪ PBS (pH 7.3–7.4) ▪ 0.01 M DTT or 0.2 M 2-ME ▪ Reagent RBCs ▪ Anti-IgG ▪ IgG-coated RBCs ▪ IgM antibody ▪ IgG antibody (optional)	For DTT and 2-ME: ▪ Test tubes ▪ Pipettes ▪ 37°C water bath/incubator ▪ Calibrated timer ▪ Calibrated serologic centrifuge ▪ Agglutination viewer For 2-ME only: ▪ Wooden applicator stick (optional) ▪ Four to six 10-inch lengths of cellulose dialysis tubing (optional) ▪ Four to six 1-liter beakers (optional)

DTT = dithiothreitol; 2-ME = 2-mercaptoethanol; PBS = phosphate-buffered saline; RBCs = red blood cells.

Use of dithiothreitol (DTT) is generally preferred over 2-ME. DTT is better at maintaining reduction of disulfide bonds, is not oxidized by air, is a water-soluble solid, and has a milder odor.[3] DTT is a dithiol; it has two reactive sulfhydryl groups (R-SH) rather than one, like 2-ME. Pirofsky and Rosner[4] stated that the additional sulfhydryl group allows for better reduction of disulfide bonds. Effectiveness of DTT was comparable to 2-ME, having identical results in 97 percent of sera tested, and DTT required a weaker concentration. Furthermore, the cyclic structure of the oxidized form of DTT with its two hydroxyl groups (-OH) on the middle carbons allow for more stability and milder odor (Figs. 2 and 3).[4] Freeman et al.[5] did report more effective dissociation of IgM antibodies with 0.2 M 2-ME than with 0.01 M DTT when comparing titration results.

Indications

Determination of the immunoglobulin class of an antibody to a red blood cell (RBC) antigen aids in the assessment of risk

Procedural Steps for Preparation

1. Mix equal volumes of plasma/serum with 0.01 M DTT or 0.2 M 2-ME in a test tube (TEST) for patient and each control.
2. Mix equal volumes of plasma/serum with PBS in a test tube (DILUTION CONTROL) for patient and each control.
3. For DTT: Incubate both sets of tubes at 37°C for 30–60 minutes.
4. For 2-ME: Incubate both sets of tubes at 37°C for 15 minutes.
5. For 2-ME: Optional step if testing with anti-IgG: Dialyze 2-ME–treated plasma/serum and dilution control.
   a. Add TEST to dialysis tubing (knotted at one end) and then knot the open end for patient and each control.
   b. Add DILUTION CONTROL to dialysis tubing (knotted at one end) and then knot the open end for patient and each control.
   c. Add each dialysis tubing to its own beaker filled with PBS; allow to dialyze overnight.
   d. Remove dialysis tubing and blot dry.
   e. Cut each tubing and transfer contents to a clean test tube.

Compiled from Freedman et al.[5] and Fung et al.[12]
DTT = dithiothreitol; 2-ME = 2-mercaptoethanol; PBS = phosphate-buffered saline.

Procedural Steps for Testing

1. Add four drops of treated plasma/serum to one drop appropriate reagent RBCs (TEST) for patient and each control.
2. Add four drops of control plasma/serum to one drop appropriate reagent RBCs (CONTROL) for patient and each control.
3. Incubate at room temperature for 30 minutes.
4. Centrifuge and read for agglutination.
5. Incubate at 37°C for 30–60 minutes.
6. Centrifuge and read for agglutination.
7. For incomplete antibodies, wash four times with saline and add two drops anti-IgG.
8. Centrifuge and read for agglutination.
9. Add IgG-coated RBCs to negative reactions.

Titration Studies

1. Prepare master serial dilutions of TEST and DILUTION CONTROL in PBS.
2. Add specified volume of each dilution of TEST and DILUTION CONTROL to labeled tube.
3. Add appropriate reagent RBCs.
4. Incubate at room temperature for 30 minutes.
5. Centrifuge and read for agglutination.
6. Incubate at 37°C for 1 hour.
7. Centrifuge and read for agglutination.
8. For incomplete antibodies, wash four times with saline, and add two drops anti-IgG.
9. Centrifuge and read for agglutination.
10. Add IgG-coated RBCs to negative reactions.

Compiled from Pirofsky and Rosner[4] and Olson et al.[9]
RBCs = red blood cells; PBS = phosphate-buffered saline.

of HDFN in pregnant women, since only IgG antibodies cross the placenta. Some antibodies to RBC antigens, like anti-M, have been described in case reports as being composed of IgM, IgG, or both classes. Therefore, assessment of HDFN risk may require determination of immunoglobulin class. For example, Mohd Nazri et al.[6] reported a severe case of recurrent fetal loss ($G_{10}P_{2+7}$) due to anti-M. Smith and Beck[7] used DTT treatment to determine whether examples of saline-agglutinating anti-M were IgM or IgG, and they found that 78 percent of these anti-M retained reactivity after treatment. Selected samples in this group were confirmed to be IgG antibodies by column chromatography. Likewise, determining that an antibody is solely of the IgM class can reduce the number of interventions during pregnancy. Jain et al.[8] reported that a non-hydropic baby was born to a woman of the Bombay phenotype, whose anti-H was nonreactive after DTT treatment.

Sulfhydryl treatment of plasma allows for the titration of IgG antibodies when both IgM and IgG antibodies are present.[9] This scenario allows relative amounts of IgG versus IgM antibody to be determined.[10]

Reesink et al.[11] compared titers of untreated, neutralized, and 2-ME–treated group O maternal sera to evaluate the relative amount of IgM and IgG ABO antibodies and found no difference in the titer results, concluding that the antibodies were solely IgG. Additionally, DTT or 2-ME treatment of plasma or serum can be used to avoid IgM antibody reactivity that may mask the presence of an IgG antibody.

Materials

The optimal concentration of DTT for inactivation of IgM antibodies was determined by Freedman et al.[5] to be 0.01 M; greater concentrations of DTT (e.g., 0.02 M DTT) were ineffective, since they caused the serum to gel during incubation.

DTT may be purchased as a water-soluble solid or as a solution. Preparation of 0.01 M DTT from the solid reagent is performed by dissolving 0.154 g of DTT in 100 mL phosphate-buffered saline (PBS), pH 7.3.[10] Preparation of the liquid DTT reagent at a 0.2-M concentration requires a dilution by adding 2 mL of 0.2 M DTT to 38 mL PBS to obtain 0.01 M DTT.

Frozen storage (–20°C) of 0.01 M DTT prepared with PBS is recommended to maintain reducing ability. Olson et al.[9] observed that freezing DTT caused no loss of reactivity over several months, but refrigerated storage (4°C) resulted in deterioration of DTT in less than 1 week. Pirofsky and Rosner[4] maintained stability of 0.01 M DTT prepared with isotonic

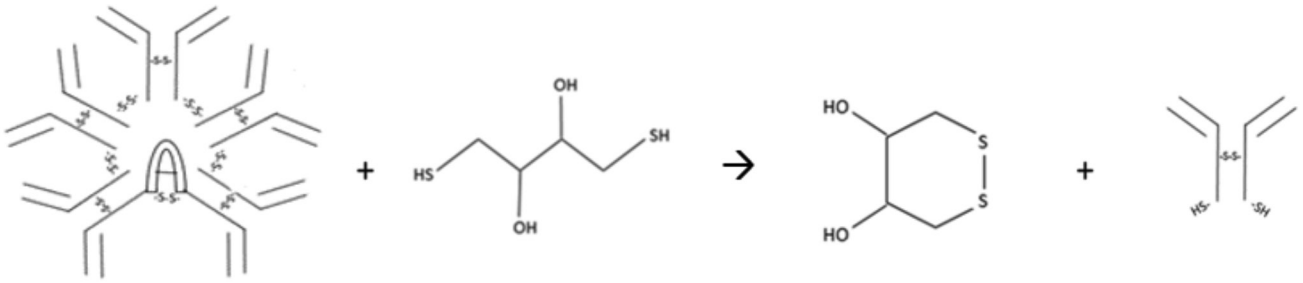

Fig. 1 Reduction of IgM by dithiothreitol.

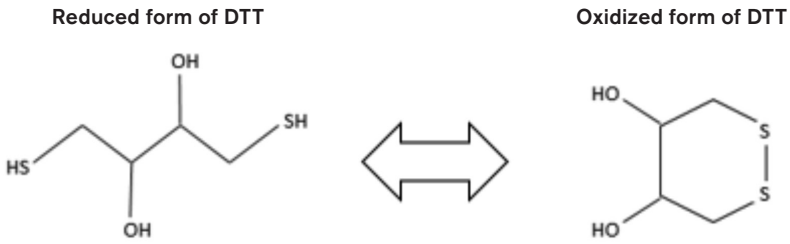

Fig. 2 Reduced and oxidized form of dithiothreitol (DTT).

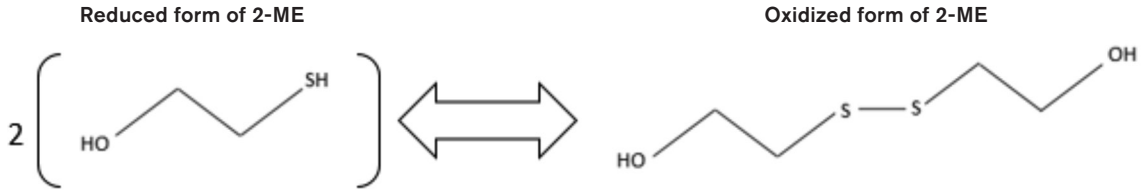

Fig. 3 Reduced and oxidized form of 2-mercaptoethanol (2-ME).

saline for up to 6 months when refrigerated or frozen, but when using DTT prepared with PBS, the reducing ability was lost within 14 days when refrigerated.

2-ME may be purchased in a solution. Preparation of 0.2 M 2-ME requires a dilution by adding 0.2 mL of 1 M 2-ME to 0.8 mL PBS, pH 7.4.

Preparation

DTT-Treated Plasma/Serum

In a test tube labeled "TEST," add an equal volume of 0.01 M DTT to the plasma or serum to be evaluated. In another test tube labeled "DILUTION CONTROL," add an equal volume of PBS to the plasma or serum. Mix the tubes and allow them to incubate at 37°C for 30–60 minutes, not to exceed 2 hours. Control samples should be treated and tested in parallel (see Quality Control).[12]

2-ME–Treated Plasma/Serum

In a test tube labeled "TEST," add an equal volume of 0.2 M 2-ME to the plasma or serum to be evaluated. In another test tube labeled "DILUTION CONTROL," add an equal volume of PBS to the plasma or serum. Mix the tubes and allow them to incubate at 37°C for 15 minutes.[5,13] An alternative method uses 0.1 M 2-ME and incubation at 37°C for 1–2 hours.[4,13] Control samples should be treated and tested in parallel.

Freedman et al.[5] observed false-positive reactions when 2-ME–treated plasma was not dialyzed, but Rosner et al.[14] found the validity of the test to not be affected by eliminating the dialysis step. The dialysis procedure, if desired, consists of

adding each sample to a segment of dialysis tubing. One end of the dialysis tubing should be tightly secured by knotting or tying before adding the sample. Once the sample has been added to the dialysis tubing, the other end should be secured by tightly knotting or tying. Each dialysis tube is suspended in its own beaker containing a large volume of PBS and allowed to dialyze overnight with several changes of PBS.[13] After dialysis, each dialysis tubing should be removed from the beaker and blotted dry, and the contents should be transferred to clean test tubes.[14]

Procedure

Test the DTT-treated or 2-ME–treated plasma or serum with reagent RBCs in appropriate serologic tests. For antibody identification panels, four drops of treated and control plasma should be mixed with one drop of appropriate RBCs.[5] Incubation for 30 minutes at room temperature and/or 30–60 minutes at 37°C may be performed depending on the initial phase of reactivity before treatment. If the antibody was reactive at 37°C or the antihuman globulin (AHG) phase, the treated and control plasmas should be washed four times after incubation at 37°C and observed for carry-over agglutination before the addition of anti-IgG. Add two drops of anti-IgG. Centrifuge the tubes, and read for agglutination. Add IgG-coated RBCs to all nonreactive tests.

Titration

For titrations studies, master serial saline dilutions are prepared for both the treated plasma and dilution control as per an in-house titration procedure. The specified volume of each dilution is mixed with an appropriate volume of reagent RBCs.[4] Incubate all tubes at room temperature (30 minutes) and/or 37°C (1 hour), centrifuge, and read for direct agglutination. Continue through to the AHG phase of testing, using anti-IgG after washing the RBCs four times.[4,9]

Quality Control

A dilution control should always be tested with DTT-treated or 2-ME–treated plasma or serum to ensure that the reduction or removal of antibody reactivity is not due to dilution of the antibody.[12] Control samples should be treated and tested in parallel.[12,13]

An IgM antibody control should be included in the DTT or 2-ME treatment with a dilution control to ensure that the sulfhydryl reagent is adequately reducing the disulfide bonds and removing the IgM antibody reactivity.[12] This antibody can be selected based on its reactivity in immediate spin tests. An IgG antibody control should be performed if testing will proceed to the AHG phase to demonstrate that these antibodies remain reactive after treatment.

Antigen-negative RBCs should be included when testing 2-ME–treated plasma by indirect antiglobulin to detect false-positive reactivity, which was described by Freedman et al.[5] when 2-ME–treated plasma was not dialyzed.

Interpretation

The interpretation of the test depends on the presence and strength of the reactivity when the controls react as expected (Table 1). The sample may be concluded as having only IgM class antibody present if the DTT- or 2-ME–treated plasma or serum has no reactivity and the dilution control remains reactive.

The sample may be interpreted as having IgG antibody or a mixture of IgG and IgM antibodies present if both the treated plasma or serum and the dilution control remain reactive.[12,13] Titration studies may be performed to determine the relative amount of each immunoglobulin class. If the titer of the treated plasma or serum is the same as that of the dilution control, it

Table 1. Interpretation of titration results using DTT- or 2-ME–treated serum/plasma

Sample	Titer*	Interpretation
Test (DTT or 2-ME + plasma/serum)	0	IgM
Dilution Control (PBS + Plasma)	16	
Test (DTT or 2-ME + plasma/serum)	16	IgG
Dilution Control (PBS + plasma/serum)	16	
Test (DTT or 2-ME + plasma/serum)	4	IgG + IgM
Dilution Control (PBS + plasma/serum)	16	
Test (DTT or 2-ME + plasma/serum)	0	Invalid
Dilution Control (PBS + plasma/serum)	0	

Adapted from Fung et al.[12]

*The absolute titer endpoints will vary depending on the specific antibody tested. The relationship between the titer endpoint of the treated plasma as compared with that of the dilution control is the critical determination of the immunoglobulin class(es) present.

DTT = dithiothreitol; 2-ME = 2-mercaptoethanol; PBS = phosphate-buffered saline.

can be concluded that there is only IgG antibody present. If the dilution control has a higher titer than the DTT- or 2-ME–treated plasma or serum, it can be concluded that there is a mixture of IgG and IgM antibodies or partial inactivation of IgM antibodies.[12,13]

The test is considered invalid if the dilution control is nonreactive, indicating that the antibody was diluted and is too weak to show antibody reactivity.[12,13] The test should also be considered invalid if the IgM and/or IgG control antibodies treated and tested in parallel do not give the expected results.

Limitations

Gelling of the serum or plasma may occur during sulfhydryl treatment when the concentration of DTT or 2-ME is too high or the test has been incubated too long.[5] Gelled samples should not be tested, since the overtreatment may result in denaturation of all serum proteins, including IgG antibodies.[12]

Sulfhydryl reagents may also weaken Kell system antigens if the reagent is not removed by dialysis before testing, because the structure of the Kell glycoprotein has disulfide bonds. It is recommended that thiol-treated plasma or serum is not used when investigating KEL blood group system antibodies, since false-negative or lower titer results may occur.[12]

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