

Video Article

Methods for Quantitative Detection of Antibody-induced Complement Activation on Red Blood Cells

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Abstract

Antibodies against red blood cells (RBCs) can lead to complement activation resulting in an accelerated clearance via complement receptors in the liver (extravascular hemolysis) or leading to intravascular lysis of RBCs. Alloantibodies (e.g. ABO) or autoantibodies to RBC antigens (as seen in autoimmune hemolytic anemia, AIHA) leading to complement activation are potentially harmful and can be - especially when leading to intravascular lysis - fatal¹. Currently, complement activation due to (auto)-antibodies on RBCs is assessed *in vitro* by using the Coombs test reflecting complement deposition on RBC or by a nonquantitative hemolytic assay reflecting RBC lysis¹⁻⁴. However, to assess the efficacy of complement inhibitors, it is mandatory to have quantitative techniques. Here we describe two such techniques. First, an assay to detect C3 and C4 deposition on red blood cells that is induced by antibodies in patient serum is presented. For this, FACS analysis is used with fluorescently labeled anti-C3 or anti-C4 antibodies. Next, a quantitative hemolytic assay is described. In this assay, complement-mediated hemolysis induced by patient serum is measured making use of spectrophotometric detection of the released hemoglobin. Both of these assays are very reproducible and quantitative, facilitating studies of antibody-induced complement activation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51161/>

Introduction

Antibodies against red blood cells (RBCs) can be induced by transfusion of RBCs expressing an antigen which is not present on recipient RBCs. These allo-antibodies can cause severe acute hemolytic transfusion reactions due to complement activation upon the following transfusion⁵. In auto-immune hemolytic anemia (AIHA), patients have auto-antibodies against their own RBCs. This leads to accelerated clearance of the cells via the interaction of IgG bound to RBCs with Fcγ-receptors on phagocytes in the spleen and/or in case of auto-antibodies able to activate complement via complement receptors in the liver^{6,7}. Fulminant complement activation resulting in intravascular hemolysis is rare but often fatal. Accelerated, complement mediated RBC destruction induced by either allo- or autoantibodies results in acute anemia and hence potentially fatal tissue hypoxia. The auto-antibodies in AIHA are classified in warm and cold antibodies, depending on the optimal temperature they bind to RBCs (37 °C or lower, respectively). The warm antibodies are usually of IgG isotype and the cold antibodies of the IgM isotype^{8,9}. AIHA can be secondary to e.g. lymphoproliferative disorders, connective tissue diseases, solid tumors, infections or drugs, but in 50% of the cases AIHA is idiopathic⁹.

Detection of gammaglobulins (e.g. IgG or IgM) and complement bound to patient's RBCs is performed by means of a semiquantitative direct antiglobulin (Coombs) test (DAT). In the DAT patient RBCs are incubated with anti-IgG or anti-C3d. Occurrence of RBC agglutination demonstrates the presence of attached complement components or IgG binding. Detection of allo- or autoantibodies in the patient's serum is performed by means of the indirect antiglobulin test (IAT). In the IAT, bromelain-treated test RBCs are incubated with patient serum, washed and then incubated with anti-human IgG. In case RBCs have been sensitized with anti-RBC IgG present in the patient serum agglutination will occur. IgM antibodies to RBCs will directly lead to agglutination upon incubation of bromelain-treated test RBCs with patient serum. RBC agglutination in the direct Coombs test or in the IAT is visually assessed either by eye in a test tube or by loading the sample on a small Sephadex column separating agglutinated and single RBCs by size¹.

Another frequently used technique to measure complement activation on RBCs is the hemolytic assay¹, in which the capacity of patient serum to induce (complement-mediated) hemolysis of bromelain-treated RBCs¹⁰ is assessed. The test is noted as positive when the supernatant after centrifugation is stained red due to released hemoglobin and considered to be negative if it remains colorless. Both the antiglobulin test and the hemolytic assay are semiquantitative, since the highest serum dilution is denoted in which the test is still positive.

The antiglobulin test and the hemolytic assay are robust assays that are routinely used in diagnostics. Since these assays are semiquantitative and dependent on the experience of the technician performing the assay they are not suitable to study subtle differences of complement

activation on RBCs, as needed when evaluating the efficacy of complement inhibitors. Therefore, we developed two quantitative assays to determine the complement activation by (auto-)antibodies to RBCs, which we will describe in this paper.

First, we developed an assay to measure the deposition of activation fragments of complement C3 and C4 on RBCs (**Figure 1A**). In this assay, human bromelain-treated type-0 RBCs are incubated with heat-inactivated patient serum (anti-RBC antibody source), fresh AB serum (complement source) and anti-C5 monoclonal antibody (Eculizumab). During this incubation, C3 and C4 deposition will occur if the patient serum contains complement activating anti-RBC antibodies. In order to prevent RBC lysis by downstream complement activation a blocking anti-C5 monoclonal antibody is added. Next, C3 and C4 deposition on the RBCs is detected by FACS using fluorescently labeled monoclonal antibodies or Fab-fragments reacting with C3 and C4, respectively. Gating on single RBCs is important to ensure the reliability of the results. Advantages of this technique include that a small volume of patient material is required, complement activation at an early stage of the cascade is assessed and the method is reproducible and quantitative. An additional advantage of looking at both C3 and C4 is that a distinction can be made between the classical and lectin pathway activation (both C3 and C4 deposition) and the alternative pathway activation (only C3 deposition). The use of bromelain-treated RBCs instead of untreated RBCs increases the sensitivity of the assay.

The second assay is based on the currently used hemolytic assay (**Figure 1B**). Human bromelain-treated type-0 RBCs are incubated with heat-inactivated patient serum (anti-RBC antibody source) and fresh AB serum (complement source). If complement is activated by patient anti-RBC antibodies, dose dependent RBC lysis will occur, leading to release of hemoglobin. The amount of released hemoglobin is quantified by measuring its absorbance at 414 nm in the supernatant after spinning down the intact and fragmented RBCs. The absorbance correlates with the amount of occurred hemolysis. In contrast to the currently used assay, this protocol allows for an objective, quantitative value of hemolysis that is very reproducible and not dependent on the person interpreting the assay.

An application of these methods has been described in ¹¹, where the potential use of C1-inhibitor as complement inhibitor in AIHA was studied.

Protocol

1. Preparation of Bromelain-treated Red Blood Cells

1. Wash 0-typed red blood cells 3x with 1x PBS (centrifugation at 664 x g for 2-5 min).
2. Incubate 1 volume of packed RBCs with two volumes of a 0.5% bromelain solution for 10 min at 37 °C.
3. Wash the cells 3x with 1x PBS.
4. The cells can be stored as a 3% solution at 4 °C in 1x PBS for at least one week.

2. C3 and C4 Deposition Analysis on RBCs by FACS

1. Heat-inactivate the required amount of patient serum (or other anti-RBC antibody source) by heating the sample for 30 min at 56 °C.
2. Wash bromelain-treated RBCs three times in VBG⁻ (5 mM Veronal, 150 mM NaCl, 0.05% gelatin pH 7.4) and resuspend them in VBG⁺⁺ (VBG⁻ + 2 mM MgCl₂ + 10 mM CaCl₂) to give a 0.5% solution.
3. Pipette the following components into a round-bottom plate with a glass pearl (for proper mixing): 25 µl 0.5% RBCs, 37.5 µl fresh AB serum (complement source) and 37.5 µl 200 µg/ml anti-C5. Anti-C5 is expensive and might be difficult to obtain. One could consider using C5-/C6-/C7- or C8-deficient serum instead of AB serum and anti-C5.
4. Add patient serum per well to a final concentration of 0.1-33%. Correct for differences in serum concentration per well due using heat-inactivated AB serum. Add VBG⁺⁺ to get a final volume of 150 µl/well. Close plate with ELISA foil.
5. Incubate 1.5-2 hr at 37 °C while shaking.
6. Wash the RBCs three times with PBS + 0.5% BSA (centrifugation at 664 x g for 2 min).
7. Add fluorescently labeled anti-C3 and anti-C4 monoclonal antibodies or their Fab fragments (to reduce agglutination) in PBS + 0.5% BSA to a final concentration of 1 µg/ml. Here custom developed anti-C3-Alexa Fluor 488 and anti-C4-Alexa Fluor 647 monoclonal antibodies are used; the choice of these fluorescent labels allows simultaneous detection of C3 and C4 by FACS.
8. Incubate 30-45 min at room temperature with gentle shaking.
9. Wash RBCs 3x with PBS + 0.5% BSA.
10. Resuspend RBCs 150 µl in PBS + 0.5% BSA and pipette them into a new plate (to get rid of the glass pearls before FACS analysis).
11. Perform FACS analysis. Separate single cells from doublets in a scatter plotting FSC-A; set the gate on the single RBCs; select the appropriate detection channel for the fluorescence signals (e.g. FITC and APC).
12. Quantify results using the median fluorescence intensity.

Note: It is possible to use an isotype control (e.g. fluorescently labeled anti-IL6 mouse IgG1). However, it is recommended to include a true negative control e.g. by incubating RBCs without patient serum (only AB serum) or without a complement source (heat-inactivated AB serum and heat-inactivated patient serum), and then stain these with the same anti-C3-Alexa Fluor 488 and anti-C4-Alexa Fluor 647 antibodies. This gives a more valid control than an isotype control in the case of red blood cells ¹², although these different controls typically give the same, negative outcome.

3. Quantitative Hemolytic Assay

1. Heat-inactivate the required amount of patient sera (or other anti-RBC antibody source) by heating the sample 30 min at 56 °C.
2. Wash RBCs 3x with VBG⁻ and once with VBG⁺⁺.
3. Pipette the following components into a round-bottom plate with a glass pearl (for proper mixing): 35 µl 3% RBCs, 25 µl fresh AB serum (complement source) and 1-50% patient serum. Correct for differences in serum concentration using heat-inactivated AB serum. Add VBG⁺⁺ to get a final volume of 150 µl/well. Close plate with ELISA foil.
4. Incubate at 37 °C for 1.5-2 hr with shaking.

5. Centrifuge plate at 664 x g for 5 min with reduced deceleration (e.g. deceleration to full stop in 2-3 min).
6. Carefully pipette 90 μ l supernatant in a new microtiter plate. It is important at this step to prevent air bubbles and to prevent to take along intact cells.
7. Measure A414/690 in a suitable spectrophotometer.
8. Express the lysis as a percentage of the lysis of a sample RBCs with pure water.

Representative Results

Figure 2A shows a representative scatter plot for RBCs. Suitable gating for single RBCs can be seen in the FSC-W - FSC-A plot (P1). Usually around 95% of the RBCs fall within this P1 gate (single cells), but when a high percentage of patient serum is used, this can drop to 70-80%, especially when anti-RBC IgM is present in the patient serum.

Representative results for the effect of AIHA patient serum on C4 deposition are shown in **Figure 2B** and on C3 deposition in **Figure 2C**. As can be seen in these figures, more patient serum leads to more complement deposition, as would be expected. Curiously, the complement deposition occurs in two distinct positive peaks. This is probably not caused by heterogeneity in the RBC population, since the RBCs are drawn from a single donor. We are still investigating the cause of this phenomenon. **Figures 2D** and **2E** demonstrate the reproducibility of the C4 (**Figure 2D**) and C3 (**Figure 2E**) deposition assays. They also show the wide variation in complement deposition capacity of the various AIHA patient samples.

A representative result of the hemolytic assay with an AIHA patient serum sample containing auto-antibodies to RBCs can be seen in **Figure 3A**. Normally, a titration with serum sample yields a nice, reproducible curve. Patient sera vary considerably in complement activating capacity; therefore perform a titration of each patient serum. Samples without patient serum usually give a background around 10-15% due to absorption by the serum sample used as complement source. Therefore, care should be taken to use a high enough patient serum concentration to obtain a signal that is substantially above this background. With a suitable complement inhibitor as anti-C5, the hemolytic signal can be titrated away as shown in **Figure 3B**.

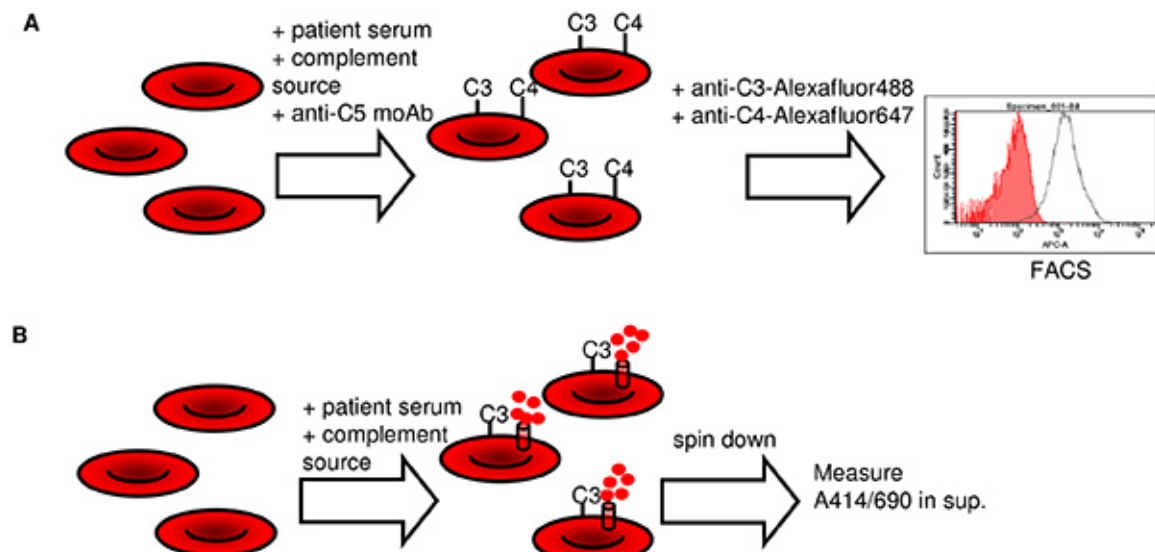


Figure 1. Schematic overview of the C3- and C4 deposition assay (A) and the quantitative hemolytic assay (B). In short, in the C3- and C4 deposition assay, complement deposition induced by AIHA patient serum is measured, while in the hemolytic assay hemolysis induced by AIHA patient serum is detected. [Click here to view larger image.](#)

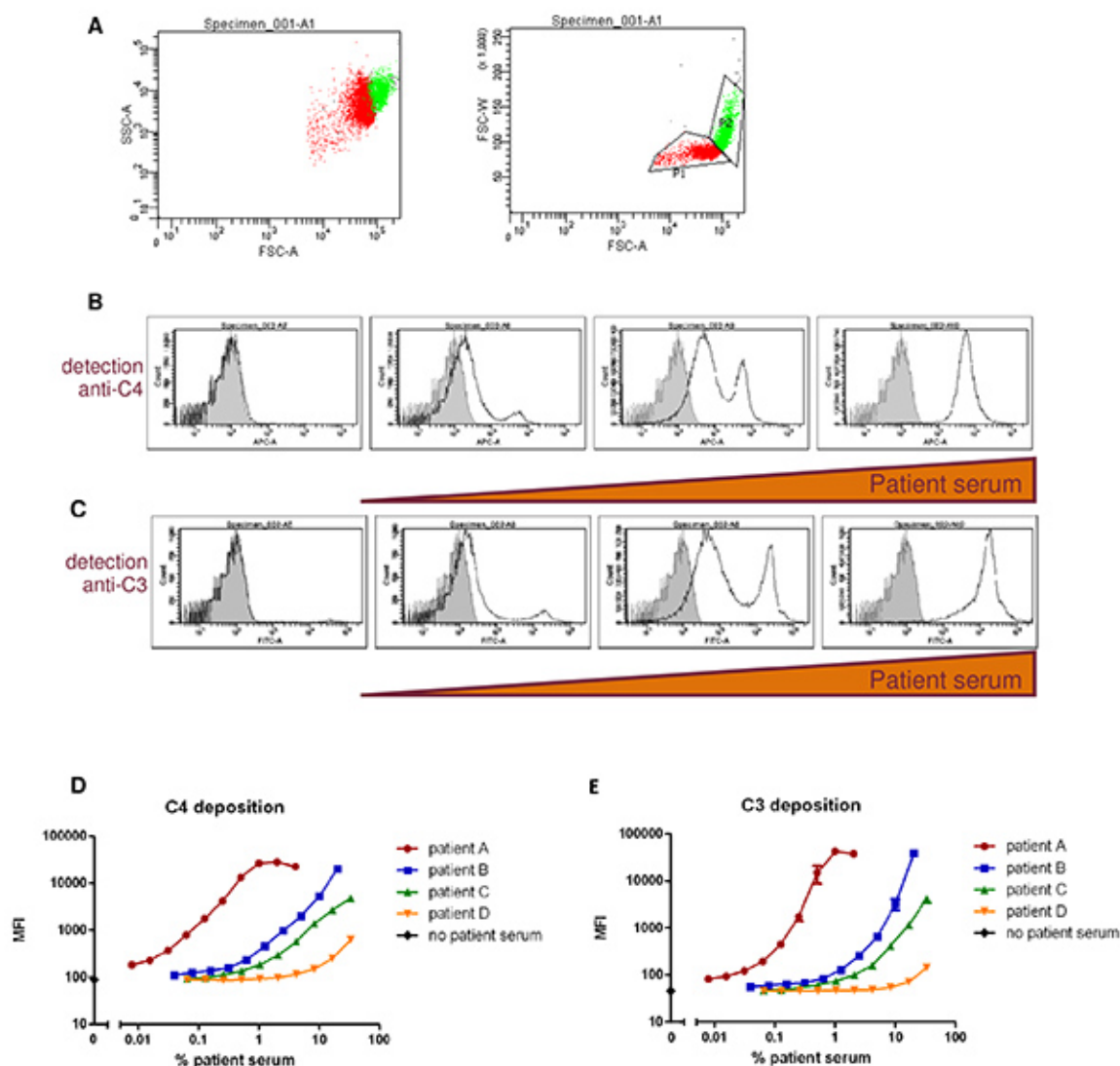


Figure 2. Representative results for C3 and C4 deposition assay. **A)** Suggested gating strategy. Shown here is how to select only for the single RBCs (P1, red). The agglutinates are shown in green (P2). **B)** The effect of a titration of AIHA patient serum on C4 deposition. The isotype control (anti-IL6 mouse IgG1) is shown as solid grey, while the sample is shown in black. Increasing the amount of patient serum leads to an increase in the C4 deposition. **C)** Similar as **B)** but now with detection for C3 deposition. **D)** C4 deposition FACS results depicted in a graph showing the reproducibility of the assay and showing the considerable differences between serum samples from different patients. The Y axis represents the median fluorescence intensity. **E)** Similar as **D)** but now for C3 deposition. [Click here to view larger image.](#)

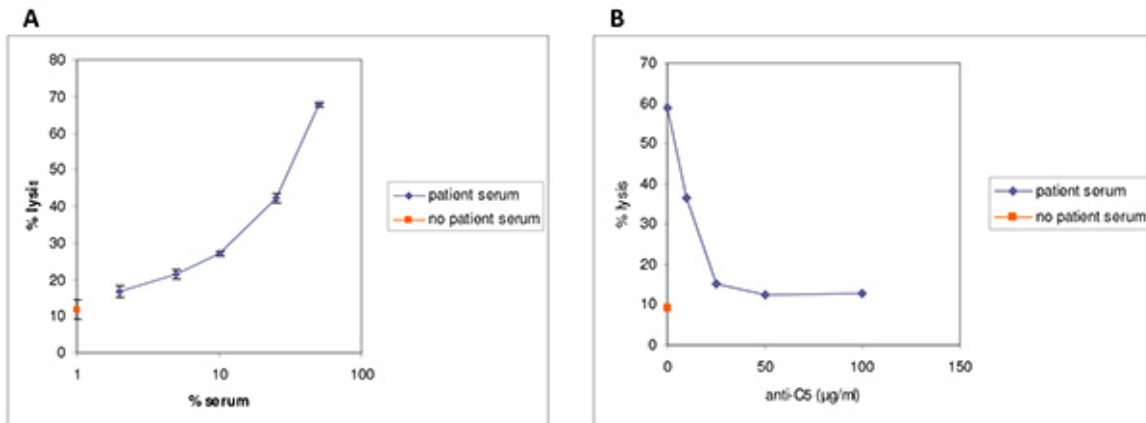


Figure 3. Representative results for the quantitative hemolytic assays. A) AIHA patient serum titration in the hemolytic assay, showing that the lysis increases reproducibly with the patient serum concentration. No lysis occurs with healthy donor serum (in orange; negative control). **B)** A suitable complement inhibitor (anti-C5 in this case) can abrogate complement-mediated lysis. Shown here is a titration of anti-C5 while the AIHA patient serum was kept at a constant concentration. [Click here to view larger image.](#)

Discussion

The above assays are reproducible and robust. They are easy to perform and it is possible to perform them with many samples at the same time (in a 96-well plate). Hence, this approach would also be suitable for a fully automated system, e.g. an ELISA robot system. In contrast to the currently used techniques, these assays are quantitative and this will help e.g. in the study of the effect of complement inhibitors. Moreover, the interpretation is objective, which is an improvement over the currently used assays.

Both assays have one critical step. Important in the FACS analysis is to gate on the single RBCs, because agglutinated RBCs will give an artificially high signal, since they are counted as one event. It is even better to prevent agglutination by using low patient serum concentrations, since low concentrations still give good signals. In the hemolytic assay, it is crucial to carefully transfer the supernatant after incubation into a new plate, since carry-over of RBCs will lead to an unreliable signal (as will air bubbles). It is recommended to use 0-typed RBCs in both assays to prevent confusion with ABO mismatch complement activation.

Due to their reliability and robustness these assays are suitable to study the efficacy of complement inhibitors for AIHA as demonstrated in reference¹¹, in order to detect quantitatively subtle differences in complement activation. Due to their sensitivity these assays may detect complement activation in patients where complement activation on RBC is strongly suggested but not detected by the routine assays, such as in Coombs-negative AIHA, though this statement still requires verification. They can also be used to determine complement activating capacity of allo-antibodies (induced by e.g. blood transfusion or in a RhD⁻ mother bearing a RhD⁺ child) which might help the clinician to predict the clinical behavior of a detected alloantibody.

Disclosures

The authors have nothing to disclose.

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