The serum complement system: a simplified laboratory exercise to measure the activity of an important component of the immune system

Jordan E. Inglis, Kimberly A. Radziwon, and Gregory D. Maniero

Biology Department, Stonehill College, Easton, Massachusetts

Submitted 25 July 2007; accepted in final form 4 August 2008

Inglis JE, Radziwon KA, Maniero GD. The serum complement system: a simplified laboratory exercise to measure the activity of an important component of the immune system. Adv Physiol Educ 32: 317–321, 2008; doi:10.1152/advan.00061.2007.—The immune system is a vital physiological system that affords animals protection from disease and is composed of innate and adaptive mechanisms that rely on cellular and dissolved components. The serum complement system is a series of dissolved proteins that protect against a variety of pathogens. The activity of complement in serum can be determined by its ability to lyse red blood cells in vitro. Here, we describe a modification of a standard complement hemolysis assay that makes an interesting and informative laboratory exercise suitable for a variety of courses including physiology.

red blood cells; immune system; hemolysis; CH₅₀; heat labile

THE IMMUNE SYSTEM is a fascinatingly complex aspect of vertebrate biology that is of the utmost importance to the health, survival, and fitness of most animals, certainly all vertebrates. The relationship of the immune system to familiar human diseases makes it an aspect of biology that readily captures the interest of beginning to advanced students. The initial exposure of students to the immune system often occurs in a first-year college biology course and is usually rather superficial, but the distinctions between innate and acquired immunity and between cellular and dissolved (or humoral) aspects of both will usually be introduced. This laboratory investigation of complement activity will demonstrate valuable immunological and physiological concepts and is designed for upper-level undergraduate college courses in physiology or immunology. The system known as complement consists of >30 plasma and cell surface proteins that function to help protect an organism from pathogens (15). The complement system is fairly well conserved throughout most of the phylum Chordata, and many of the components are closely related and may have arisen through gene duplications (3, 6, 13). Unlike antibodies, complement proteins are not specific for particular immunogens and do not improve in affinity or increase in concentration with repeated exposure and, as such, are defined as components of innate immunity (5).

The ability of cell-free human serum to kill pathogenic microbes was discovered at the end of the 19th century (14). It was observed that bacteria are killed by lysis and that this lytic ability of serum was enhanced with prior immunization with the target bacteria. When heated, serum was able to agglutinate but not lyse the bacteria with which the host had been immunized. Lytic ability was restored upon mixing heated serum with fresh serum, even if the additional serum was from a nonimmunized animal. These experiments demonstrated that the ability of cell-free immune serum to lyse bacteria is due to at least two factors: one heat labile and present in the serum of naïve and immunized animals, and the other a heat-stable component that is only present after immunization. The heat-stable, adaptive factor able to agglutinate the bacteria was recognized as antibody. The heat-labile, innate serum fraction was eventually given the term complement and found to be a series of dissolved proteins.

There are three separate but related pathways of complement activity: the classical pathway addressed in this experiment, the alternative pathway, and the lectin pathway. Each of the complement pathways are a protease cascade, a phenomenon that encompasses a variety of familiar physiological processes including blood clotting. The three pathways have disparate routes of initiation but share downstream effector functions essential for inflammation and pathogen clearance (2). The alternative and lectin complement pathways function independently, without the involvement of adaptive immunity. In contrast to these other pathways, the classical complement pathway is activated in the presence of specific antibodies. In this way, the classical pathway can be envisioned as a link between innate and adaptive immunity, and demonstration of this pathway provides the opportunity for discussion of both facets of the immune system.

The specific functions and details of the classical complement cascade, which are relevant to the experiment presented here, are described in many physiology textbooks (and in any basic immułogy text). Briefly, the classical pathway is activated by antibody that has bound to the surface of an invading cell. The membrane-bound antibody activates the first complement component, which activates eight additional complement proteins. The ultimate result is the formation of what is known as the membrane attack complex, a series of proteins that forms a pore in the membrane, resulting in the lysis of target cells.

The experiment detailed here describes a simple method to demonstrate and quantify the activity of serum complement proteins. Vertebrate red blood cells (RBCs; also known as erythrocytes) contain large amounts of the cytoplasmic respiratory pigment hemoglobin, which is easily detected upon release from the cell. Lysis of RBCs turns the experimental diluent red, and the intensity of the red color, which equates to the amount of hemoglobin released, can be measured photometrically. For the experiment described here, briefly, antibody is collected from a rabbit that had been highly immunized with cell membranes (stroma) of sheep RBCs (SRBCs). The antibody is purified and, when reconstituted in the laboratory, does not contain any other components of the host animal’s serum, including complement proteins. Whole SRBCs are then incubated with the antibody solution, again in the absence of any complement proteins.

Address for reprint requests and other correspondence: G. D. Maniero, Biology Dept., Stonehill College, 320 Washington St., Easton, MA 02357 (e-mail: gmaniero@stonehill.edu).

1043-4046/08 $8.00 Copyright © 2008 The American Physiological Society 317

Downloaded from http://advan.physiology.org/ by 10.220.33.3 on June 19, 2017
Complement binds to the highly conserved constant region of antibody, allowing complement proteins to bind and be activated by immunoglobulins originating in a different species. For this experiment, the sensitized (antibody-coated) erythrocytes are subsequently incubated with guinea pig serum as the source of complement. Lysis of the SRBCs by activated complement results in the release of hemoglobin into the buffer. Target SRBCs that were not lysed by complement are separated from the supernatant by centrifugation, and the intensity of the red color in the supernatant is quantified by measuring the absorbance of the solution at 540 nm. The measured absorbance correlates with the amount of hemoglobin released due to complement-mediated hemolysis (rupture of RBCs).

### MATERIALS AND METHODS

**Buffers.** Complement hemolysis occurs at physiological pH and has been analyzed in a variety of buffers. The buffer employed in the experiment described here uses triethanolamine (TA) and will be referred to as TA-complement hemolysis buffer (TA-CHB). Additionally, the complement cascade is highly dependent on Ca$^{2+}$ and Mg$^{2+}$, and these cations are included in the buffer. Stock TA-CHB is prepared as a 5× solution. To make 1 liter of 5× TA-CHB, combine solution A and solution B. Solution A contains 42.66 g NaCl (0.73 M) and 4.134 g TA·HCl (0.02 M, T1502, Sigma) dissolved in ~900 ml of deionized (di)H$_2$O. Solution B contains 4.06 g MgCl·6H$_2$O dissolved in 10 ml diH$_2$O plus 6.0 ml of 1 M CaCl$_2$; adjust to 20 ml in diH$_2$O (1.0 M MgCl$_2$ and 0.3 M CaCl$_2$).

Add 2.5 ml of solution B to solution A, bring the buffer to a final volume of 1.0 liter in diH$_2$O, and mix thoroughly. This 5 × solution should be stored at 4°C and kept for up to 6 mo. Within 24 h of use, dilute to a 1× solution in diH$_2$O. To eliminate the possibility of contamination that may lyse erythrocytes or interfere with the complement cascade, filter the working TA-CHB through a 0.2-μm filter before use.

To determine the percent of complement-mediated hemolysis, a positive control with 100% lysis of all target erythrocytes must be used. An ammonia lysis buffer can be used, which can be prepared as follows: dissolve 9.09 g NH$_4$Cl, 0.041 g EDTA, and 0.100 g KHC$_2$O$_3$ and bring the solution to 1 liter in diH$_2$O; adjust the solution to pH 7.4 with NaOH, and store it at room temperature.

**Sheep erythrocytes.** SRBCs can be washed and diluted a day in advance of the laboratory exercise. SRBCs in Alsever’s solution (CS 11125, Colorado Serum) are sent as a 20% erythrocyte suspension in Alsever’s solution. SRBCs should be stored at 4°C and used within 2 wk of arrival. The complement hemolysis assay is most sensitive when the absorbance of the 100% hemolysis well is at or near 0.500. In a 96-well flat-bottomed plate, this absorbance is obtained with a 0.500, and, if not, the RBC solution can be diluted accordingly. A small deviation from a 4% solution or an absorbance of 0.500 (±0.050) does not affect the results of this exercise in any appreciable way.

**Antibody and serum.** On the same day of the experiment, dilute reconstituted antibody to SRBC (rabbit anti-SRBC stroma, S8014, Sigma) by 1:100 in TA-CHB and store it at 4°C until use. Dilute reconstituted guinea pig sera (used as the source of complement, S1639, Sigma) by 1:50 in TA-CHB and leave it at 4°C until use. Fresh rat, mouse, human, or even frog serum are all excellent sources of complement.

To compare the heat-labile nature of complement components with the temperature stability of antibodies, a portion of the serum dilution can be heated in a 50°C water bath for 20 min to effectively eliminate complement activity. [C2 is the most heat labile of the components of the mammalian classical complement pathway (8)]. A portion of the antibody solution, before the sensitization of erythrocytes, can be heated in the same way with little detriment to complement-mediated hemolysis in the presence of normal serum. Storage of the serum dilution overnight or even for several hours at room temperature will greatly reduce hemolytic activity, whereas antibody will remain intact under the same conditions.

**In-class experiment.** Provide TA-CHB, washed 4% SRBC solution, diluted antibody, and diluted serum on ice. Sensitize erythrocytes with antibody by adding equal volumes of SRBC solution and antibody dilution. Incubate for 10 min at room temperature and return to ice bath. In a round-bottomed, 96-well microtiter plate, add the solutions in the order shown in Table 1.

Incubate the completed plate at 30°C for 30 min. During incubation, resuspend RBCs by gently tapping the side of the plate every 10 min. At the end of this incubation period, add 50 μl of TA-CHB to each well and centrifuge the plate at 200 g for 10 min at 4°C. Remove 200 μl of the supernatant from each well, and gently resuspend the pelleted RBCs, and transfer this to the corresponding wells of a 96-well flat-bottomed ELISA plate. Visual observation of the supernatants will clearly demonstrate increasing hemolysis with increasing serum volume as well as allow comparison of different experimental conditions (see below). To compute mathematical values for complement activity, read the absorbance of this plate using an ELISA plate reader at 540 nm.

This laboratory exercise is easily completed in a 3-h period and requires careful but straightforward use of a micropipettor. In addition to typical laboratory equipment, this exercise requires some specialized equipment, namely, a centrifuge with a rotor for 96-well plates and a microplate reader capable of reading absorbance at a wavelength of 540 nm.

**Computation of complement activity.** Absorbance values are used to determine the activity of complement in the serum. Complement activity can be calculated as the volume of serum that is required to cause the same hemolytic activity as a standard volume of serum. To determine the absorbance corresponding to 50% hemolysis, absorbance readings are plotted on a graph, and the absorbance of 50% hemolysis is determined. The absorbance of 50% hemolysis can be calculated using the following formula:

\[ \text{Absorbance of 50% hemolysis} = \frac{\text{Absorbance of 100% hemolysis} + \text{Absorbance of 0% hemolysis}}{2} \]

Once the absorbance of 50% hemolysis is determined, the absorbance of each sample can be calculated and compared to the absorbance of 50% hemolysis to determine the complement activity.

**Table 1. Distribution of components in a round-bottomed 96-well plate**

<table>
<thead>
<tr>
<th>Order of Addition (from first to last)</th>
<th>1. TA-CHB, μl</th>
<th>2. Diluted serum, μl</th>
<th>3. Lysis buffer, μl</th>
<th>4. Coated RBCs, μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row A</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row B</td>
<td>183</td>
<td>17</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row C</td>
<td>175</td>
<td>25</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row D</td>
<td>167</td>
<td>33</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row E</td>
<td>150</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row F</td>
<td>133</td>
<td>67</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Row G</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>50</td>
</tr>
</tbody>
</table>

TA-CHB, triethanolamine-complement hemolysis buffer; RBCs, red blood cells.
lyse 50% of the erythrocytes in a standardized sample, a volume known as CH₅₀. To compare the relative complement activity of different serum samples, it is useful to determine the number of CH₅₀ volumes in a milliliter of serum, or CH₅₀/ml. Complement activity expressed in this way is highly dependent on the experimental conditions, including RBC density, incubation temperature, antibody source, etc., and only samples examined at identical conditions can be compared directly.

To determine CH₅₀/ml, one must first determine the relative absorbance (RA) of each well to eliminate any contribution of the plate, buffer, or noncomplement hemolysis, as follows:

\[ RA = \text{absorbance at 540 nm of the well} - \text{absorbance of the 0% lysis well (row A; 0 µl serum)} \]

Next, determine the percent RBC lysis of each well as follows:

\[ \text{Percent RBC lysis} = \frac{\text{RA of the well}}{\text{RA of the 100% lysis well (row G)}} \times 100 \]

Complement hemolysis as a function of serum volume produces a sigmoid curve (Fig. 1A) that is best analyzed by log-transforming variables before analysis. Before log transformation, percent lysis (the y-value) should be expressed as follows: \( y/100 - y \). Because 50% lysis would result in \( \log(y/100 - y) = 0 \), CH₅₀ can be determined as the x-intercept on a simple line graph constructed using log(serum volume) as the independent variable and \( \log(y/100 - y) \) as the dependent variable (Fig. 1B).

The y-intercept gives CH₅₀ in microliters of diluted serum. To determine CH₅₀/ml of pure serum, multiply the intercept value by the dilution factor as a whole number (in this case, the dilution = 50) and divide 1,000 by the resultant value to calculate CH₅₀/ml. As an alternative to graphing, the x-intercept can be determined using a regression equation. An Excel spreadsheet for calculating CH₅₀/ml from raw absorbance measurements is available from the author upon request.

**RESULTS AND DISCUSSION**

The experiment outlined here was derived from a similar procedure carried out in individual tubes and read on a spectrophotometer (4, 7). Protocols for the evaluation of complement activity very often use a 0.15 M barbitone-buffered saline (4, 7) containing barbital and barbital sodium, both of which are regulated in the United States as class IV narcotics by federal and state drug enforcement agencies. The exercise detailed here eliminates the use of regulated components. Additionally, this assay, using microtiter plates, utilizes far less material, solutions, samples, and time than its test tube counterpart. Although this exercise requires transfer of small volumes with a micropipettor, it provides valuable practice of an essential laboratory skill. Because the students are performing a linear dilution, pipetting errors are easily detected.

Complement hemolysis is often determined as the ability of a volume of serum to lyse 50% of 5 \( \times 10^7 \) cells (16). Since this experiment uses smaller volumes than traditional assays, it also employs a smaller number of target cells (2–3 \( \times 10^7 \)) per reaction, and the calculated CH₅₀/ml may be different than published results from assays employing a larger number of cells. This difference is of no importance to this exercise since we are comparing samples side by side under identical conditions. Similarly, the direct comparisons employed here make exact dilution of the erythrocyte solution unnecessary since all rows use the same solution. Batches of SRBCs can differ considerably since the erythrocytes from different individual animals differ in their sensitivity to complement hemolysis. These differences are of no concern in this experiment, again, because all sample are tested simultaneously. It is this inherent flexibility that makes this exercise highly suitable and convenient for use as a college laboratory exercise.

Despite all of the differences from traditional assays using test tubes, the results from this experiment in our laboratories have consistently yielded values of ~600–1,000 CH₅₀/ml when native guinea pig serum is employed as the complement source and values of 0–50 CH₅₀/ml with heat-denatured sera. Representative data are shown in Table 2. It is readily apparent from the data shown in Table 2 that native serum is superior to heated serum for complement-mediated hemolysis of target erythrocytes. It is also apparent from the sample data that heated antibody is nearly as efficient as untreated antibody for the initiation of the classical complement pathway. Taken together, these results demonstrate that complement components are heat labile, yet immunoglobulins are heat stable.

Figure 1 shows control data from Table 2 (experiment A: native antibody and native sera). Figure 1A shows the logarithmic relationship of complement hemolysis to serum volume, and Fig. 1B shows the linear relationship of the transformed data.
Teaching in the Laboratory

A SERUM COMPLEMENT LABORATORY EXERCISE

Table 2. Representative data from experiments using native antibody and native guinea pig sera, native antibody and heat-denatured sera, and heat-treated antibody and native sera

<table>
<thead>
<tr>
<th>Volume, ml (x)</th>
<th>Raw Absorbance at 540 nm</th>
<th>Adjusted Absorbance</th>
<th>Percent Lysis (y)</th>
<th>Log(x)</th>
<th>Log(y100 – y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.035</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
<td>-2.07</td>
</tr>
<tr>
<td>17</td>
<td>0.039</td>
<td>0.004</td>
<td>8.85</td>
<td>1.23</td>
<td>-1.99</td>
</tr>
<tr>
<td>25</td>
<td>0.045</td>
<td>0.010</td>
<td>2.13</td>
<td>1.23</td>
<td>-1.99</td>
</tr>
<tr>
<td>33</td>
<td>0.060</td>
<td>0.025</td>
<td>5.32</td>
<td>1.52</td>
<td>-1.25</td>
</tr>
<tr>
<td>50</td>
<td>0.100</td>
<td>0.065</td>
<td>13.83</td>
<td>1.70</td>
<td>-0.79</td>
</tr>
<tr>
<td>67</td>
<td>0.240</td>
<td>0.205</td>
<td>43.62</td>
<td>1.83</td>
<td>-0.11</td>
</tr>
<tr>
<td>100% lysis</td>
<td>0.505</td>
<td>0.470</td>
<td>100</td>
<td>1.00</td>
<td>0</td>
</tr>
</tbody>
</table>

Experiment A: native antibody and native sera

| Titer          | 623.68 CH50/ml |

Experiment B: native antibody and heat-denatured sera

| 0              | 0.034                    | 0.000               | 0                 | 0      | -2.07         |
| 17             | 0.039                    | 0.005               | 1.00              | 1.00   | -1.99         |
| 25             | 0.039                    | 0.005               | 1.00              | 1.40   | -1.99         |
| 33             | 0.040                    | 0.006               | 1.20              | 1.52   | -1.10         |
| 50             | 0.042                    | 0.008               | 1.61              | 1.70   | -1.79         |
| 67             | 0.044                    | 0.010               | 2.01              | 1.83   | -1.69         |
| 100% lysis     | 0.532                    | 0.498               | 100               | 1.00   | 0             |

Experiment C: heat-treated antibody and native sera

| Titer          | 0.53 CH50/ml |

CH50 is the volume of serum that is required to lyse 50% of RBCs in a standardized sample; CH50/ml is the number of CH50 volumes in a milliliter of serum.

This experiment has been successfully employed in the laboratory portions of two very different classes at Stonehill College: Immunology (BI 409) and Vertebrate Physiology (BI 312). Both immunology and physiology students found the exercise and associated lecture helpful for their understanding of complement and the interactions and differences between innate and adaptive immune functions in vertebrates. The experiment was easily performed in the 3-h time allotment, and the downtime associated with the longest incubation was used productively (in these cases, students used the time to examine vertebrate blood smears and learn to identify different subsets of leukocytes).

Like all experimental protocols, there are pitfalls with this experiment that should be avoided. Due to the heat-labile nature of complement proteins, it is essential that the serum be thawed and handled on ice, used immediately after the thawing and diluting, and never refrozen. The students performing the experiment need to exercise care not to aspirate SRBCs when transferring the supernatant to the new plate. In the event that this occurs, the solution can be returned to the original well and the plate can be centrifuged again. This laboratory exercise, which assesses complement activity, is suitable for many levels and types of classes and can be further modified to pursue additional questions suitable for advanced courses. Because of the evolutionarily conserved nature of vertebrate complement components and antibody Fc regions, serum from a variety of animals, including amphibians (1, 11, 12), can be employed in this exercise. This facet of the experiment can be used as a springboard for discussions of the evolution of the immune system, the differences between Fc and Fab regions of the immunoglobulin molecule, or the different subtypes of antibodies that activate the classical complement pathway. The antibody-dependent nature of the classical pathway can be demonstrated by running a series of wells that use SRBCs incubated with either a nonspecific antibody or with buffer alone, acting as an additional negative control.

As shown in Table 2, comparisons can be made between experiments performed with native antibody and serum, heat-treated antibody and native serum, or native antibody and heat-treated serum. Students can use all of these conditions to demonstrate the temperature stability of antibody compared with the temperature-labile nature of complement as well as the dependence on antibody for classical pathway hemolysis. Alternately, separate groups can be given different antibody-serum combinations as unknowns, and the results can be compared between groups.

The nature of this exercise also allows for the introduction of concepts as universal as the temperature sensitivity of proteins or amplification and control in protein cascades. This complement assay could also be modified to demonstrate the effect of anticomplementary factors other than heat, including preincubation of serum with cobra venom, which activates complement by cleaving C3 into C3a and C3b. The importance of Ca²⁺ and Mg²⁺ can be demonstrated by adding wells that use a buffer that lacks these important cations and would not allow for complement-mediated hemolysis.

The complement system is important for warding off many human diseases. Additionally, deficiencies in or inappropriate
activation of the complement system can cause a variety of illnesses in humans, making it an excellent physiological component to discuss and demonstrate in the context of a student laboratory exercise.

ACKNOWLEDGMENTS

The authors thank the students, faculty, and administration of Stonehill College for the help and support.

GRANTS

This work was partially funded by a seed grant (to G. D. Maniero) from the Declining Amphibian Population Task Force (2005).

REFERENCES