Comparison of Three Different Methods for Measuring Classical Pathway Complement Activity

TROY D. JASKOWSKI,1* THOMAS B. MARTINS,1 CHRISTINE M. LITWIN,1,2 AND HARRY R. HILL1,2

Associated Regional and University Pathologists Institute for Clinical and Experimental Pathology1 and the Department of Pathology, Pediatrics and Medicine, University of Utah School of Medicine,2 Salt Lake City, Utah 84108

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The complement system plays an important role in host defense against infection and in most inflammatory processes. The standard 50% hemolytic complement (CH50) assay is the most commonly used method of screening patient sera for functional activity of the classical complement pathway. Our objective in this study was to compare two newer methods (the enzyme immunoassay and the liposome immunoassay) to a commercial CH50 assay for measuring total classical complement activity. We conclude that both newer methods compare well with a CH50 assay and are equally sensitive in screening routine clinical sera.

Assessing the functional integrity of the complement system (classical pathway) has been accomplished in the clinical laboratory by traditional 50% hemolytic complement (CH50) assays for many years. Hemolytic assays based on the Mayer method (6) require the interaction of titered complement components in patient sera with antibody-sensitized sheep erythrocytes in solution. The titer at which 50% hemolysis occurs (CH50 unit) is proportional to the functional activity of the classical pathway in the serum. Only recently have different methods been developed for measuring total classical complement activity. The objective of our study was to compare two newer methods (the enzyme immunoassay [EIA] and the liposome immunoassay [LIA]) to a commercial CH50 assay for measuring total classical complement activity.

The levels of complement activity in sera from cord blood and neonates are approximately 50% or less of that in normal adults (1, 3, 8, 11, 12). Moreover, using the CH50 method, sera that are deficient (homozygous) in a single classical pathway component show very low or no hemolytic activity, whereas sera with low levels of a single component (heterozygous) have hemolytic activity approximately 50% of the normal level (2, 10). Inherited deficiencies of early complement components are frequently associated with rheumatic disorders, recurrent infection, and various immune abnormalities (2, 4, 9, 10). The most common component deficiency is that of C2 (heterozygous), which has a frequency of 1% in the general population (4). Three hundred and thirty-one patient sera sent to our laboratory for CH50 testing were used for comparison in this study. In addition, sera from cord blood (n = 19 samples), newborns (n = 31), and patients with known complement abnormalities (homozygous, n = 14; heterozygous, n = 3) were included in the study. Sera were donated by Patricia C. Giclas, National Jewish Medical Center, Denver, Colorado; Hajime Kitamura, The Center for Adult Diseases, Osaka, Japan; and by our C2-deficient family at the University of Utah Medical Center, Salt Lake City, Utah. All sera were stored at −70°C until tested. Sera giving discrepant results between assays were retested for result verification.

The CH50 assay (Diamedix, Miami, Fla.) utilizes sensitized sheep erythrocytes in solution and is a simplified variation of the Mayer method (6). The degree of cell lysis is proportional to the total classical complement activity present in the serum. Interpretation of CH50 units is as follows: <100, low; 100 to 300, normal; and >300, high.

The LIA (Waco Chemicals USA, Richmond, Va.) utilizes dinitrophenyl (DNP)-coated liposomes that contain the enzyme glucose-6-phosphate dehydrogenase. When serum is mixed with the liposomes and a substrate containing anti-DNP antibody, glucose-6-phosphate, and nicotinamide adenine dinucleotide, activated liposomes lyse, and an enzymatic colorimetric reaction occurs which is proportional to total classical complement activity. Interpretation of LIA units is as follows: <23, low; 23 to 60, normal and >60, high. LIA testing was performed with a Hitachi 717 automated analyzer per the manufacturer’s protocol.

The EIA combines the principles of the hemolytic assay with the use of a monoclonal antibody specific for neoantigen (C5b-9 complex) produced as a result of complement activation (Incstar, Stillwater, Minn.). The amount of polymerized C5b-9 (final product) is proportional to the functional activity of C1 through C9. Interpretation of EIA units is as follows: <60, low; 60 to 140, normal; and >140, high.

Other than the kits to measure classical complement activity, no funds were derived from the manufacturers for these studies.

Statistical analysis showed the LIA (Waco) to have an agreement of 94.6%, a sensitivity of 93.2%, and a specificity of 95.0% compared to the CH50 method (Diamedix) using the 331 patient sera (Table 1). Compared to the CH50 method, the EIA (Incstar) showed 94.0% agreement, 95.9% sensitivity, and 93.4% specificity (Table 1). The EIA showed 98.2% agreement, 100.0% sensitivity, and 97.6% specificity compared to the LIA (Table 1).

In the sera from patients known to have a complement deficiency (homozygous, n = 14), all assays gave results far below their cutoff values for normal classical complement activity (Table 2). In contrast, when using sera from patients with known low levels of a complement component (heterozygous), the EIA gave values at or below the cutoff, whereas the CH50 assay and LIA indicated these sera had normal levels of classical complement activity (Table 3).

When measuring total complement activity in the 19 cord
and 31 newborn sera, the EIA showed all sera to have classical complement activity less than that of normal adults (Table 4). In contrast, the CH₅₀ assay and LIA detected normal adult levels of classical complement activity in 28% and 22%, respectively, of the cord and newborn sera combined (Table 4).

Statistical analysis showed good correlation for both the LIA and EIA compared to the CH₅₀ in screening sera for total classical complement activity in the clinical laboratory (Table 1). Moreover, the EIA and LIA showed 100.0% sensitivity compared to each other (Table 1), indicating that these assays may be more comparable than the Diamedix CH₅₀ assay when screening patient sera for total classical complement activity.

Using sera with various complement deficiencies (homozygous), all methods showed classical complement activity to be very low or undetectable in all 14 sera (Table 2). The low but detectable levels demonstrated in some sera using these methods may be due to activation of the alternative pathway or to a very low level of the component that is often present even in some homozygous deficient sera (one of our C2-deficient patients had 0.2 mg/dl of C2 while the other had an undetectable concentration). When using sera with low levels of a complement component (heterozygous), the values obtained using the EIA depicted more accurately what one would expect to find as far as functional activity in patients with heterozygous abnormalities (Table 3).

In sera from newborns and cord blood, only the EIA found all sera to have classical complement activity levels less than normal adults (Table 4). Since the EIA is dependent on the amount of neoantigen (C5b-9 complex) generated, one might expect this method to be more sensitive to the low levels of C9 found in neonatal sera (5).

The protocol for the CH₅₀ method (Diamedix) is very laborious, especially with a large number of samples. Dilutions and spectrophotometric readings are performed manually for each patient sample in this CH₅₀ assay. The LIA (Waco) has been adapted to many common automated analyzers and was the least labor intensive of the three methods. Using a Hitachi 717, LIA results for 60 sera (plus controls) are generated approximately 30 min after test initiation. The EIA (Incstar) requires serum specimens and serum diluent to be kept at 2 to 8°C while dilutions are being made. This method also requires a 37°C incubator and a 2-h total incubation time.

We conclude that the LIA and EIA showed good correlation compared to CH₅₀ (Table 1), but that both may be more sensitive than the Diamedix CH₅₀ assay when screening patient sera for total complement activity in the clinical laboratory (Table 1). It has been suggested that solid-phase assays such as EIA that use monoclonal antibodies for the detection of neoantigens may be of more value in assessing complement in patient sera (7, 13). Given the results found in the heterozygous, cord, and newborn sera, it is our opinion that the EIA has higher accuracy in detecting low to moderate depressions of complement activity, especially in the case of C9. One should remember that assays such as these measure only the functional activity of the complement components of the classical pathway and are strictly qualitative. When screening for the functional integrity of classic complement components as a whole, the sensitivity of the assay to detect slight depressions is of utmost importance. Since complement activity over time may reflect disease activity, which has been assessed in the past.

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**TABLE 1.** Comparison of the LIA, EIA, and CH₅₀ assay of 331 patient sera for total classical complement activity

| Test and level of complement activity | CH₅₀ | LIA | EIA |
|--------------------------------------|------|-----|-----|
| Low                                 | 68   | 13  |     |
| Normal                              | 5    | 245 |     |
| Low                                 | 70   | 17  | 81  |
| Normal                              | 3    | 241 | 0   |

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**TABLE 2.** Complement activity units obtained from the CH₅₀ assay (Diamedix), LIA (Waco), and EIA (Incstar) for total classical complement activity in heterozygous sera

| Homozygous deficiency | CH₅₀ assay | LIA | EIA |
|-----------------------|------------|-----|-----|
| C1                    | 0          | 0   | 0   |
| C1                    | 0          | 4   | 0   |
| C2                    | 3          | 11  | 0   |
| C2                    | 3          | 11  | 0   |
| C2                    | 0          | 0   | 0   |
| C2                    | 0          | 5   | 0   |
| C6                    | 0          | 5   | 11  |
| C6                    | 0          | 6   | 1   |
| C7                    | 2          | 10  | 10  |
| C8                    | 0          | 4   | 23  |
| C8                    | 0          | 5   | 12  |
| C9                    | 0          | 8   | 4   |
| C9                    | 0          | 11  | 0   |
| C9                    | 0          | 9   | 0   |

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**TABLE 3.** Complement activity units obtained from the CH₅₀ (Diamedix), LIA (Waco), and EIA (Incstar) for total classical complement activity in heterozygous C2 sera

| Heterozygous C2 sera | CH₅₀ assay | LIA | EIA |
|----------------------|------------|-----|-----|
| C2                   | 102        | 31  | 57  |
| C2                   | 112        | 30  | 62  |
| C2                   | 112        | 31  | 50  |

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**TABLE 4.** Results obtained by three different methods for measuring total classical pathway complement activity in cord and newborn sera

| Serum type (no. of samples) | CH₅₀ assay | LIA | EIA |
|----------------------------|------------|-----|-----|
| Low                       | 15         | 4   | 16  |
| Normal                    | 16         | 3   | 19  |
| Low                       | 21         | 10  | 23  |
| Normal                    | 23         | 8   | 31  |

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* Complement activity units for the CH₅₀ assay are as follows: <100, low; 100 to 300, normal; >300, high.
* Complement activity units for the LIA are as follows: <23, low; 23 to 60, normal; >60, high.
* Complement activity units for the EIA are as follows: <60, low; 60 to 140, normal; >140, high.
using CH₅₀ assay values, it will be necessary to validate the LIA and EIA against a CH₅₀ assay longitudinally in such disease states.

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