Standardized, Automated Procedure for Measurement of Serum Hemolytic Complement Activity

I. OLITZKY

Department of Microbiology-Immunology, Bio-Science Laboratories, Van Nuys, California 91405

Received for publication 23 May 1968

In the interest of standardizing and automating complement fixation procedures, an AutoAnalyzer manifold was designed in which the reagents are mixed in proportions similar to that of the Laboratory Branch Complement Fixation test. By passing the fixation stage, the manifold proved useful for measuring serum hemolytic complement activity (C'). As many as 21 serum samples per hour can be screened by testing two appropriate dilutions. Normal adult C' titers measured by the automated procedure ranged from 58 to 103 C'H₄₀ units per ml.

This paper describes an automated method for the measurement of total serum hemolytic complement (C'). An AutoAnalyzer manifold has been designed which, unlike those previously described for complement fixation (CF) tests and C' titrations, (6, 10), combines reagents in the same proportions as recommended by the Laboratory Branch Task Force of the National Communicable Disease Center (1). This procedure, called the Laboratory Branch Complement Fixation (LBCF) method, was devised to promulgate a standardized CF procedure that can be used with any antigen. Its use would facilitate interlaboratory comparisons of CF tests. The application of the same manifold design to antibody-detecting CF reactions is being studied.

There is much clinical interest in C' and the serum components which interact in a certain sequence to produce the hemolytic activity (2). The importance of C' measurements in diagnosis and prognosis is well documented (2, 4, 7, 8). Serum C' activity is depressed in poststreptococcal glomerulonephritis and in systemic lupus erythematosus, especially with renal involvement. Higher than normal C' levels are found in patients with inflammatory diseases. The published data of C' levels in various diseases loses some of their value because of the lack of uniformity in the measurement methods employed. The method of Mayer (5) has been used most frequently. The developers of the LBCF have related their procedure to the Mayer method and the continuity has been preserved by relating the automated procedure to the manual LBCF method.

MATERIALS AND METHODS

Manifold design. The manifold and flow diagram used are shown in Fig. 1. Fittings are labeled with the manufacturer’s designation (Technicon Co., Ardsley, N.Y.). Pump lines deliver reagents in the same proportions used in the LBCF manual titration of C'. The antigen and sample lines both pump buffer during C' titrations and may be combined into one line with a flow rate of 1.2 ml per minute. If the manifold is to be used only for C' titrations, the delay coil A, used for the fixation state of CF tests, is bypassed. The cam (30 1/1) used in the Sampler II was chosen to provide a large “wash” at the expense of a high sampling rate (“slow manifold”). The feasibility of faster sampling rates was also studied by substituting a 70 2/1 cam (45-sec sample, 7-sec wash at a rate of 70 cups per hour; “fast manifold”).

At the end of each day, lines were flushed with 2.5% urea in 0.05 N NaOH for 15 to 30 min, followed by distilled water for several hours.

Reagents. Hemolysin, sheep red blood cells (RBC), and guinea pig C' were obtained from commercial sources and all reagents prepared as described in the LBCF method (1). Sheep red blood suspensions were standardized by photometry of a hemoglobin solution prepared by lysing 1.0 ml of the cell suspension with 14.0 ml of buffer (LBCF method). A standard reading of 190 Klett units with a no. 54 filter in place is obtained from a cell concentration of 670,000 cells per mm³, as determined by hemocytometer counts.

Hemolysin and guinea pig C' titrations. Simultaneous hemolysin and C' titrations were performed by feeding into the manifold replicate sets of guinea pig C' (GPC') dilutions (four or five dilutions ranging from low to high concentration), each set separated by three-cup buffer “spacers”. Five different batches of sensitized cells (hemolysin dilutions from 1:4000 to 1:800) were pumped into the manifold, one batch for each set of C' dilutions. The change from one batch to another (starting with the most dilute hemolysin) was made during the time the buffer “spacers” were being sampled.

The “plateau” method was used for selecting the optimal amount of hemolysin, as in the LBCF pro-
cEDURE. The GPC' titer was determined from the points (dilutions and the resulting hemolysis), in that set related to the optimal hemolysin dilution. One of the C' dilutions must give a complete (maximal hemolysis), which for purposes of calculation was considered as 100% hemolysis. The other recorded values are adjusted accordingly and the percentage of hemolysis produced by each dilution in the set is converted to Y/(100 - Y) values (ratio of percentage of lysed to percentage of nonlysed cells) using tables from Mayer (5) and plotted on log-log paper (log transformation of von Krogh's equation). Our plot differs from the LBCF method in that dilutions of C' (or serum), rather than amounts, are plotted on the ordinate. Both the C'H50 end point and the slope (5) of the line were determined.

Automated titration of human serum C'. As soon as possible after removal from clot, serum samples were divided into convenient samples, which were kept at -70 C. Each sampler tray included 4 or 5 dilutions of a reference pool of guinea pig C'. From the experience gained from previous detailed titrations, dilutions were chosen to provide one dilution which would produce complete hemolysis, with the other dilutions producing hemolysis in the 10 to 90% range. During the latter part of the study, a pooled human serum was used for the quality control standard.

Serum specimens from 12 adult, normal males and 12 adult, normal females were tested. Initially, four dilutions of each serum were included in each run in a four-point assay. The dilutions were chosen to bracket the 50% hemolysis dilution, and yet stay in the 10 to 90% range. Each set of dilutions was loaded into cups, starting with the most dilute. One-cup buffer spacers were used to separate sets. After several replicate determinations, the samples were reassayed by the use of two "screening" dilutions picked to closely bracket the expected 50% hemolytic end point (two-point assay). The purpose of this modification was to assess the feasibility of assaying more samples per hour without sacrifice to precision. Titers were calculated as previously described for guinea pig C'.

Manual titrations of human serum C'. Twenty of the serum samples assayed by the automated procedure were also tested by the LBCF C' titration procedure. Titrations were carried out in a total volume of 1.0 ml, and they were calculated as described in the LBCF method. The titers were divided by five to relate them to the LBCF "master" system (total volume, 5 ml).

RESULTS

Combined GPC' and hemolysin titrations. The simultaneous titration of hemolysin and GPC' was conveniently performed by use of the manifold described above. A typical titration is shown in Fig. 2. The optimal hemolysin dilution was 1:1000, and the GPC' titer, calculated from the set associated with the optimal hemolysin dilution, was 450 C'H50 units per ml (slope = 0.19). A detailed C' titration of the same guinea pig serum pool at the optimal hemolysin dilution is
shown in Fig. 3. The titer was calculated to be 470 C'H₅₀ units per ml.

Automated human serum C' titrations. There was no significant difference between normal male and normal female serum C' levels. A normal equivalent deviate (NED) plot revealed a symmetrical distribution; a log transformation was not indicated. The mean C' titer for the 24 adults was 80.6 C'H₅₀ units per ml. One standard deviation (SD) was found to be ±9 C'H₅₀ units per ml. The normal range was calculated by "K" statistics (3) to be 58 to 103 C'H₅₀ units per ml.

Precision. Intra-run precision was excellent. There generally was a slight drop in titer between a serum specimen assayed at the beginning of the run and the same specimen assayed at the end of the run. This presumably is due to the lability of complement at room temperature. With a human serum pool measuring around 53 C'H₅₀ units per ml, the drop in titer was 5 C'H₅₀ units in the hour between the sampling of the first and second set of dilutions. Guinea pig C' dropped about 13% in 1 hr (titer of 350 C'H₅₀ units per ml to a titer of 308 C'H₅₀ units per ml).

Inter-run precision was calculated from 35 separate determinations on the same human serum pool used above, and was found to be ±8 C'H₅₀ units per ml for two standard deviations.

LBCF manual human serum C' titrations. The mean titer for the 20 normal adults (determined by the manual method) was 46.4 C'H₅₀ units per ml and, again using "K" statistics, the normal range was calculated to be 28 to 65 C'H₅₀ units per ml.

Intra-run precision was calculated from 11 separate determinations on the serum pool used above, and was found to be ±6.8 C'H₅₀ units per ml for two SD.

Comparison of two- and four-point assays. Typical four-point and two-point assays are shown in Fig. 4 for the "slow" manifold and in Fig. 5, for the "fast" manifold. Two-point assays gave C' titer results not significantly different to those obtained by four-point assays if the two dilutions chosen for assay produced hemolysis bracketing the 50% hemolytic end point. When dilutions of 1:70 and 1:100 were tested, the C' levels of normal serum samples could be calculated directly. When the two-point assay produced results that could not be plotted, it was necessary to reassay the sample, using appropriate dilutions.

Titration curve slopes. Mayer (5) and the authors of the LBCF test (1) looked with suspicion on C' titers that were the result of plots where the slopes of the titration curves were outside the limits of 0.20 ± 0.02. The developers of the LBCF test state, however, "on occasion
... slope values up to 0.28 did not affect complement titer.\(^5\) In the early experiments with the automated procedure, a range of 0.16 to 0.24 was set for "acceptable" titrations. Experimental evidence now available leads to the conclusion that, in the automated procedure, the slope of the titration curve does not necessarily reflect the quality or the quantity of C' or the quality of the titration (Table 1).

**Interaction.** With the "slow manifold", the effect of a sample with high C' activity on the next sample with low C' activity was negligible, ap-

<table>
<thead>
<tr>
<th>Table 1. Titration curve slopes and titers as related to type of assay (two- or four-point)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>P11</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P11</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P11</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The number 6, wherever appearing, indicates results of 2- and 4-point assays combined.

The measurement of total serum hemolytic C' activity may be accurately and precisely accomplished with a relatively simple AutoAnalyzer manifold. Because of the need for standardization of "serological" determinations and because of a long-range plan to use AutoAnalyzer equipment for CF assays, the manifold was designed to combine reagents in the same proportions as in the highly standardized LBCF method.

**Master volume.** The hemolytic C' titer of a guinea pig or a human serum in the automated, the LBCF, and the Mayer systems is the amount of serum necessary to hemolyze 50 percent of sensitized RBC. C' titers, as determined by the LBCF system, are higher than those determined by the Mayer system on the same samples by a ratio of 3:2 because the "master" volume Mayer titration contains 500 \(\times 10^4\) RBC, whereas there are \(333 \times 10^4\) RBC in the LBCF "master" volume.

The master-volume concept is not applicable to a continuous-flow system so long as the reactants are mixed in fixed proportions. In the "slow manifold", the ratio of the amount of C' to the total number of sensitized RBC is the same as it is in the "fast manifold". Because of the higher C' titers obtained, it is obvious that in the automated procedure C' was tested with less than the \(333 \times 10^4\) sensitized RBC present in the 5.0-ml volume of the "master" LBCF test. The mean ratio of automated titers of the normal serum specimens to manual titers of the same specimens was 1.8, with a range of \(\pm 0.6\) for two SD.

**Titration curve slopes.** In the Mayer, the LBCF, and the automated titrations, the C' titers are calculated from the plot of log X against log \((Y/1 - Y)\) using the log transformation of the von Krogh equation \((\log X = \log K + 1/n \log (Y/1 - Y))\, where X equals the amount of complement and Y equals the percent lysed. [The plot consisted of the reciprocal of serum dilutions (X) against percentage of hemolysis (100% hemolysis) on log-log paper.] Many reports dealing with the titration of C' by the Mayer method stress the importance of "acceptable slopes", the acceptable range usually stated as 0.2 \(\pm 0.02\).

The slope of the titration curve, influenced by red cell concentration, Ca\(^{++}\) concentration, and
the disproportionate loss of C⁺ activity can signal test deficiencies when sets of different dilutions of a serum are exposed to ambient or incubation temperatures (5). In this experience, however, slopes were most influenced by the ability to select serum dilutions that would produce hemolysis close to the 50% hemolysis end point. Assuming that all reagents are prepared correctly, an acceptable titration is defined as one in which the chosen serum dilutions produce hemolysis close to and on both sides of the 50% hemolysis end point. If the useful points (>10 and <90% hemolysis) are unequally distributed around the 50% end point, it is better to have the majority of points between 50 and 80% hemolysis rather than between 20 and 50%. Points for plotting can be selected after the titration (Table 1) because the C⁺ values calculated from the “good” or “bad” plots may not differ significantly. Mayer pointed out that C⁺ titers may even be calculated from single values by assuming a curve with slope of 0.2. (A table for calculating C⁺ from single values can be found on page 139 of reference 5.) This is recommended only for values between 35 and 70% hemolysis.

ACKNOWLEDGMENTS

The technical assistance of Brenda Stuber is gratefully acknowledged, as is the initial manifold design assistance of Gerald Kessler.

LITERATURE CITED

ERRATA

Standardized, Automated Procedure for Measurement of Serum Hemolytic Complement Activity

I. OLITZKY

Department of Microbiology-Immunology, Bio-Science Laboratories, Van Nuys, California 91405

Volume 16, no. 11, page 1635, column 2, line 12: Change “2/1” to “6/1.” On page 1636, line 4 of legend to Fig. 1: Change “2/1” to “6/1.”

Improving Reliability in Penicillin Assays

S. G. PATHAK

Microbiology Department, Philadelphia Pharmaceuticals and Cosmetics, Inc., Philadelphia, Pennsylvania 19131

Volume 16, no. 12, page 1941, column 2, line 4: Change “genicillin” to “penicillin.”