Comparison of a Fluorometric Method with Radial Immunodiffusion Assays for Determination of Complement Components C3 and C4

MARGOT KOELLE AND WILLIAM R. BARTHOLOMEW*
Division of Clinical Microbiology and Immunology, Erie County Laboratory, Erie County Medical Center, Buffalo, New York 14215, and Department of Microbiology, School of Medicine, and Department of Medical Technology, School of Health Related Professions, State University of New York at Buffalo, Buffalo, New York 14214

Received 23 November 1981/Accepted 29 April 1982

Measurements of patient serum complement components C3 and C4 are useful indicators of complement consumption in immune complex diseases. A fluorometric quantitative immunofluorescence system was evaluated in terms of measuring these complement components, and the results were compared with those of radial immunodiffusion assays. For comparison of the two systems, 232 patient sera were evaluated for C3, and 202 specimens were tested for C4. Analysis of the data by linear regression indicated a proportional difference between the methods. C3 and C4 concentrations measured by the fluorometric method were lower than those measured by radial immunodiffusion, especially concentrations exceeding the normal ranges. In detecting lower concentrations (<120 mg/dl for C3 and <25 mg/dl for C4), the two methods showed better agreement. Each assay system was reproducible and could be used to evaluate changes that occur in concentrations of complement components during therapeutic treatment. However, the ease in processing a large volume of specimens and the short time needed to complete the assay are advantages that make the fluorometric method more suitable than radial immunodiffusion for use in a large clinical laboratory.

Concentrations of complement components C3 and C4 in serum are used as indicators of complement consumption in immune complex diseases. Concentrations of C3 and C4 have been of particular value as an aid in the diagnosis and management of systemic lupus erythematosus. Monitoring the concentration of these serum complement components has been used to determine the effectiveness of therapeutic treatment of this disease (1, 7).

The quantitation of serum complement components is usually performed by radial immunodiffusion (RID). RID assays are based on an antigen-antibody precipitation reaction. Antigen is added to wells cut in an agarose matrix which contains an optimal concentration of monospecific antiserum. In the method of Fahey and McKelvey (6), the precipitin rings are measured within a specific time period; complete diffusion is not necessary. The protein concentrations of the specimens are determined by interpolation from the standard curve on which the diameters of the precipitin rings are plotted against the log of concentrations of the standards. One of the more recently developed test systems for the quantitation of complement components and other serum proteins is a solid-phase fluorometric assay (3, 9).

The purpose of this study was to evaluate the quantitative immunofluorescence (QIF) system for the quantitation of complement components C3 and C4 and to compare this technique with traditional assays. The QIF assay is based on the reaction of serum complement component C3 or C4 with fluorescein isothiocyanate (FITC)-labeled monospecific antibodies in a buffer solution. After a 20-min incubation, the unreacted antibodies are adsorbed onto small disks coated with C3 or C4. The disks are 6 mm in diameter and attached to the lower portion of plastic probes, which can easily be inserted into 75- by 100-mm test tubes. After adsorption of the residual labeled antibodies, the probes are washed in buffer, and the amount of fluorescence emanating from the disk is determined by insertion of the probe into the specifically designed fluorometer. The amount of fluorescence, measured in fluorescent signal units (FSU), is inversely proportional to the concentration of the complement component in the serum.
The precision of the assay systems, as well as the total time for performing each assay, is discussed in this study.

MATERIALS AND METHODS

**Serum samples.** We evaluated the QIF system by testing 232 patient serum samples for complement component C3 and 202 samples for C4, using both the fluorometric and the RID methods. The sera were randomly selected from hospital and clinic samples submitted for complement assays. Many of the specimens from clinic patients were from systemic lupus erythematosus patients who were being monitored. Normal ranges for complement components C3 and C4 were established by the QIF system, using sera of 102 healthy adults. These sera were donated by healthy laboratory personnel.

Blood specimens were held at room temperature for 1 h after being drawn to allow a clot to form and then held at 4°C for 1 h so that the clot would retract. At this point, the specimens were immediately centrifuged at 500 × g for 10 min, divided into portions, and stored at 70°C until assayed.

**Fluorometric analysis.** (i) **Instrumentation.** The FIAx fluorometer (International Diagnostic Technology, Santa Clara, Calif.) was the instrument used for the QIF assays. It is equipped with a tungsten light source. The excitation wavelength is 475 nm, and the emission is read at 540 nm. A specifically designed stage holds the plastic probes, which have attached, cellulose-like disks on which the reactions take place. A microcomputer interfaced with the fluorometer was used to obtain a standard curve and calculate the values for each specimen. A horizontal shaker was utilized to agitate the tubes during the washing and various incubation periods. It is designed to hold test tube racks at a 45° angle and operates at 200 oscillations per min at a distance of 2.5 cm. All dilutions were performed with a pipette diluter (model 1500; Cavro Instruments, Los Altos, Calif.). This instrument can be adjusted from 5 to 50 μl for the delivery of reagents or serum samples, and the diluent may be set from 0.25 to 1.0 ml.

(ii) **Reagents and method.** Test kits for the determinations of C3 (β,C/β,A) and C4 were obtained from International Diagnostic Technology. The procedure described by the manufacturer was followed without modification.

To start the complement assay for C3, we mixed 50 μl of a 1:10 dilution of standard or patient serum with 50 μl of FITC-labeled monospecific antiserum in 500 μl of phosphate-buffered saline (pH 7.4). The mixture was incubated for 20 min. The tubes were placed on the horizontal shaker at the beginning of the incubation period. During the same time, the reaction surfaces of the tubes were coated with the C3 complement component by insertion of the plastic probes with attached disks into 500 μl of the component reagent supplied with the kit. After 10 min, the probes were removed and placed into a second row of tubes containing phosphate-buffered saline (pH 7.4) for a wash. The disks were then ready to be used in the assay and were placed into the incubated serum-antiserum mixture. Residual, monospecific, FITC-labeled antibodies to C3 were absorbed by the C3-coated disks during a 20-min incubation. The disks were then washed in phosphate-buffered saline (pH 7.7), and the amount of fluorescence attributable to residual labeled antibody was determined with the fluorometer; the intensity was expressed in FSU. The standard curve was based on the FSU of four standard sera supplied in the kit. FSU being inversely proportional to the concentrations of C3 in the standard sera.

The levels of C3 in patient serum samples were determined by interpolation from the standard curve. For the C4 assay, 20 μl of a 1:13.5 dilution of standard or patient serum was mixed with 20 μl of the FITC-labeled monospecific antiserum in 500 μl of phosphate-buffered saline (pH 7.4). The incubation periods, as well as the procedure for coating the disks with C4, were the same as those used for the C3 assay. The determination of FSU and standard curves were similar to the determination of C3.

**RID.** Test kits were obtained from Meloy Laboratories, Inc., Springfield, Va. The procedure employed was that given on the insert included in each kit. The incubation time for the C3 determinations was 18 h at 4°C, and that for the C4 determinations was 22 h at 37°C. The antiserum used in the agarose for the measurement of serum C3 by RID was shown to react with β,C and β,A. The diameters of the precipitin rings were measured with a vernier caliper and a viewer-magnifier (National Instrument Laboratories, Inc., Rockville, Md.). To obtain a standard curve, we plotted the concentration of each serum standard (log [ordinate]) against the diameter of each precipitin ring (abscissa) on semilog paper. For each new lot, we established a standard curve by plotting three values for each control serum. Each time the assay was performed, high and low standards were included on each plate and checked against the values of the standard curve to ensure that the kit was functioning satisfactorily. Patient and standard sera were assayed in the same manner. After diffusion, the precipitin rings were measured, and the concentration of complement component in each patient serum was determined by interpolation from the standard curve.

RESULTS

**Complement concentrations found in sera of healthy individuals.** The normal ranges for the complement components C3 and C4, as measured by the fluorometric system, were based on the analysis of 102 sera. The frequency distribution curves, plotted in increments of 0.5 standard deviation, showed a bimodal distribution. The Kolmogorov-Smirnov test, used to determine whether the observed data followed a normal distribution, indicated no significant deviation for C3 (P > 0.05) but did show significant deviation for C4 (0.01 < P < 0.05). On the basis of the mean ± two standard deviations, we calculated the normal range for C3 to be 72 to 170 mg/dl (mean, 121 mg/dl), and that for C4 to be 12 to 46 mg/dl (mean, 29 mg/dl). The range for C4, calculated from nonparametric data (excluding 2.5% of the highest values and 2.5% of the lowest), ranged from 13 to 45 mg/dl.

**Comparison of the fluorometric system with RID.** The fluorometric system was compared...
FIG. 1. Quantitation of C3: comparison of QIF and RID. The statistical analysis is shown in Table 1. Line of best fit: \( y = 45.7 + 0.421x \); \( n = 232 \); \( s_{yx} = 19.2 \). The highest point not plotted but included in calculations was 137, 368 (y, x).

with RID in terms of measuring C3 and C4. A total of 232 serum samples, 91 of which were from patients with systemic lupus erythematosus, were analyzed for C3. For the C4 evaluation, 202 serum samples were analyzed, 91 of which were from patients with systemic lupus erythematosus.

The results for C3 obtained by the fluorometric assay were plotted against RID results (Fig. 1), using the RID assay as the reference method. The data for C4 are plotted in Fig. 2. The statistical analyses of C3 and C4 by linear regression are summarized in Table 1.

For C3, the range of concentrations plotted varied from 25 to 368 mg/dl. The slope of the regression line for C3 was 0.421, and the intercept was 45.7. Considering that a slope of 1.0, an intercept of 0, and minimal random error indicate an ideal bivariate relationship, the data for C3 show a proportional as well as a constant difference between the two assay systems. Results of QIF were lower than those of RID. The random error was 19.2 mg/dl, as defined by the standard deviation for distribution of the observed y values above and below the regression line (\( s_{yx} \)). The coefficient of correlation was 0.788.

For C4, the plotted values ranged from 4 to 73 mg/dl (Fig. 2). The line of best fit was \( y = 5.3 + 0.694x \). The slope of 0.694 indicates proportional difference, and the intercept (5.3) relates to some degree of constant difference in C4 measurement between the two assay systems. At levels of 25 mg/dl, both methods showed good agreement. The random error, \( s_{yx} \), was 6.2 mg/dl; the correlation coefficient, which relates to the fit of the values to the regression line, was 0.855. Three outliers were excluded from the data (4 \( s_{yx} \)). Considering the large number of data, the elimination of obvious outliers is appropriate (4).

**Precision of QIF and RID assays.** (i) RID plates. The run-to-run precision of the RID method for quantitating C3 and C4 was determined by inclusion of high and low standards for C3 and C4 in each assay. These standards had to give ring diameters that fell within a predetermined range to be considered as having acceptable sensitivity and precision. The coefficient of variation (CV) was calculated from the concentrations (mg/dl) obtained for the high and low standards for C3 and C4. The CV for C3, determined by using the high standard in 61 determinations, was 8.24%. The CV for C3, based on results of the low standard in 59 determinations, was 4.27%. In terms of the run-to-run precision of the C4 assay, the CV for the high standard (58 determinations) was 6.74%, and that for the low standard (56 determinations) was 8.76%.

(ii) QIF assay. The within-run precision of the C3 assay was determined by evaluation of a standard serum eight times within a test run. The CV was 3.1%. In terms of the C4 within-run precision, the CV was 7.4%, determined on the
basis of 10 determinations. The run-to-run precision for the C3 and C4 determinations was determined with a freshly thawed portion of a serum pool (stored at -70°C) in each run. The CVs were calculated from a total of 38 determinations for each C3 and C4. The CV for C3 was 9.3%, and that for C4 was 9.7%.

**Time comparison of QIF and RID assays.** The quantitation of C3 and C4 in 10 serum samples by the QIF system was accomplished in 3 h, including dilution of the sera, performance of the assay (which includes four standards and two dilutions of the control serum per test run), and reading and reporting the results.

The RID assay of C3 and C4 in 10 serum specimens took approximately 1 h. After 18 h of incubation for C3 and 22 h for C4, the precipitin rings are read, the concentrations are calculated, and the results are reported. The latter three tasks took a total of 2 h; thus, the total time needed to quantitate C3 and C4 in 10 serum specimens by RID was approximately 3 h. However, the time from receipt of the specimen to submission of a report would be a minimum of 21 to 25 h for RID, whereas the total time for the QIF system would be approximately 3 h.

**DISCUSSION**

The QIF and RID systems were compared in terms of the quantitation of complement components C3 and C4. A total of 232 clinical specimens were analyzed for C3, and 202 specimens were analyzed for C4. Analysis of the data by linear regression indicated a significant proportional difference for both C3 and C4 (slopes of 0.421 and 0.694, respectively) and some degree of constant difference. QIF values for C3 and C4 tended to be lower than RID values. This was especially true for C3 in concentrations above 160 mg/dl: the concentrations determined by RID were almost twofold greater than those determined by QIF. The correlation coefficients were 0.788 for C3 and 0.855 for C4, which

**TABLE 1. Quantitation of complement components C3 and C4: statistical comparison of the QIF and RID assays by linear regression analysis**

<table>
<thead>
<tr>
<th>Component</th>
<th>No. of specimens tested</th>
<th>Conc (mg/dl)</th>
<th>QIF $y$ (mg/dl)</th>
<th>RID $x$ (mg/dl)</th>
<th>Slope</th>
<th>Intercept</th>
<th>$S_{yx}$</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>232</td>
<td>25-368</td>
<td>114.6</td>
<td>163.6</td>
<td>0.421</td>
<td>45.7</td>
<td>19.2</td>
<td>0.788</td>
</tr>
<tr>
<td>C4</td>
<td>202</td>
<td>4-73</td>
<td>25.7</td>
<td>29.2</td>
<td>0.694</td>
<td>5.3</td>
<td>6.2</td>
<td>0.855</td>
</tr>
</tbody>
</table>
indicates that the correlations were 62.1 and 73.1%, respectively.

For the determination of serum complement component C3, RID and QIF are designed to measure serum fractions \( \beta_1C \) and \( \beta_1A \). \( \beta_1A \) being a breakdown product of \( \beta_1C \). Of the three antigenic determinants present on \( \beta_1C \), only the A determinant is shared by \( \beta_1A \). It has been found that aged serum samples deplete more monospecific antibodies to the A determinant than are depleted by the amount of \( \beta_1C \) in fresh serum (5, 10). Davis et al. (5) have reported that when serum samples are held at 37°C for up to 7 days, the ratio of the A determinant in aged versus fresh sera is 1.56. These researchers found no appreciable difference when serum samples were held at 5°C. An autoanalyzer was used for the determination of \( \beta_1C \) and \( \beta_1A \). The fact that the serum specimens assayed in our study were stored at -70°C until assayed rules out the possibility of any appreciable breakdown of \( \beta_1C \) from the time the specimen was received to the time the assay was performed.

Bruver and Salkie (2) compared C3 values obtained by using the Hyland model PDQ nephelometer and the RID method of Mancini et al. (8). For the RID method, the researchers chose a shortened incubation of 16 h at room temperature, after which the diffusion rings were measured. Two kits were used for the comparison of nephelometric and RID. In the analysis of the data by linear regression, the researchers reported higher values obtained by nephelometric analysis than by RID. However, we observed that values for C3 obtained by the fluorometric method were lower than those obtained by RID. The correlation coefficient reported by Bruver and Salkie (nephelometric assay versus RID) was similar to the value we obtained for the fluorometric assay versus RID, 0.79 (Fig. 1).

When monitoring patients for C3 and C4, both the fluorometric and RID assays were capable of detecting a change in complement component concentration.

In terms of the within-run precision of QIF, the CV for C3 was 3.1%, and that for C4 was 7.4%. Values for within-run precision of RID have been reported by Stevens et al. (9). The CV for the run-to-run precision of the RID assays, based on the concentrations (milligrams per deciliter) of high and low standards, ranged from 4.27 to 8.24% for C3 and from 6.74 to 8.76% for C4. In terms of the run-to-run reproducibility of QIF, the CVs, based on a serum pool, were 9.3 and 9.7% for C3 and C4, respectively.

The QIF method is relatively easy to perform, and values may be reported within the same day that the specimen is received. The QIF system provides a high degree of precision in the determination of serum complement components C3 and C4.

Both methods are suitable for quantitating patient serum complement components in clinical laboratories. The QIF system, however, provides a reliable alternative method for the quantitation of C3 and C4. The results may be reported on the same day that the specimen is received, whereas the RID method requires a minimum of 2 days before the results can be reported.

ACKNOWLEDGMENT

We thank Helen Lees, Department of Medical Technology, State University of New York at Buffalo, Buffalo, for her guidance with the statistical analysis.

LITERATURE CITED