Anti-IgG Binding Test to Assay Circulating IgG-Containing Immune Complexes from Polyethylene Glycol Precipitates

Stanley S. Levinson,¹ Janice O. Goldman,¹ and Carolyn S. Feldkamp²

We describe a simple approach for assaying immune complexes from serum by using anti-IgG as the indicator after a three-step extraction procedure with polyethylene glycol. Analysis of the data indicates that assays of such extracts for immune complexes by absorbance nephelometry, kinetic light scatter, and immunoradiometric techniques correlate well. For 116 samples, results by absorbance nephelometry correlated (r = 0.86) with those by the C1q-binding test. The present assay and the Raji cell test were more sensitive than the C1q-binding test (p < 0.001) for detecting increased concentrations of immune complexes in 29 samples from patients with immune-complex-type diseases. The basic approach we describe may lend itself to broad applications for use with various immun assay techniques.

Additional Keyphrases: absorbance nephelometric, kinetic nephelometric, and immunoradiometric assays compared · C1q-binding test · Raji cell test · systemic lupus erythematosus

Measurement of circulating immune complexes (IC)³ is useful in diagnosis and follow-up of many diseases (1). C1q-binding and Raji cell tests are most commonly used to measure IC, but the complex preparation of reagents needed limits the performance of these tests to relatively few laboratories.

We developed a simple assay for IC based on absorbance nephelometry with anti-IgG as the indicator after extraction with polyethylene glycol (PEG) of IC from serum (2). Success depends on adequate removal of monomeric IgG by washing it from the PEG precipitate before the assay, and on use of a high-precision technique for measuring the IC: we used absorbance nephelometry with a double-beam spectrophotometer.

The C1q-binding test measures the number of sites on IC to which complement is not bound, whereas the Raji cell test measures the sites to which complement is bound. Together, these assays are assumed to reflect the total IC status of a patient. Surprisingly, we have found that our assays, which are a measure of IgG in IC from PEG precipitates, correlate highly with the C1q-binding test but not with the Raji cell test. Here we present data indicating that it may be necessary to reconsider whether two test IC profiles are clinically necessary, or whether a single test could do as well, especially in light of recent findings of interference by antinuclear antibodies in the Raji cell assay (3, 4).

Materials and Methods

Materials

For absorbance nephelometry, we used a double-beam spectrophotometer with digital read-out (Model 25; Beckman Instruments, Fullerton, CA 92634). Kinetic light scatter was measured with an immunochemistry system (ICS, Beckman Instruments). Data reduction for the immunoradiometric assay (IRMA) was performed with a Hewlett-Packard 85 microcomputer with an Inform program for IRMA-spline (Corning Medical, Medfield, MA 02042).

Heat-aggregated gamma-globulin (AHG) was prepared from human globulin, Cohn Fraction II, as previously described (2).

To prepare calibrators and controls, we diluted aliquots of AHG with Tris buffer (20 g of bovine serum albumin (BSA) and 10 mmol of Tris per liter of isotonic saline, pH 7.4 ± 0.1) or pooled serum, respectively.

Sera were obtained by centrifugation. One portion of each sample was stored at −80 °C for assay by us, and another portion was sent either to Scripps Reference Laboratory, La Jolla, CA 92037, or to SmithKline Clinical Laboratories, King of Prussia, PA 19406, for Raji cell and C1q-binding assays.

¹¹²I-labeled anti-IgG was prepared by a modification of the Chloramine-T method (5), and purified by passage through a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). The radioactivity in the labeled product was between 20 and 30 mCi/L.

Procedures

PEG extraction of IC from serum. This procedure, described previously (2), is briefly as follows. In duplicate, add 0.2 mL of each serum sample to 0.6 mL of PEG solution in PBS (per liter: 33 g of PEG and 10 mmol of sodium phosphate in isotonic saline, pH 7.4 ± 0.1). Let the mixture stand overnight at 4 °C, then collect the precipitate by centrifugation (2000 × g, 30 min) at 4 °C the next morning. Wash the pellet by resuspending it in 0.8 mL of ice-cold PEG (per liter: 25 g in 15 mmol/L PBS, pH 7.4 ± 0.1), shake vigorously, and collect the precipitate a second time by centrifugation at 4 °C. Resuspend the pellet in 0.2 mL of Tris-BSA buffer (as in Materials above) and precipitate overnight with 0.6 mL of the 33 g/L PEG solution. After each centrifugation step, aspirate the supernate and discard. Resuspend the final pellet in 0.2 mL of Tris buffer for immediate assay. The pellets easily form a homogeneous suspension in this buffer.

Alternatively, one can complete the extraction in a single morning by substituting a second wash for the second precipitation. For this, add BSA, 25 g/L, to the 25 g/L PEG wash solution. After the initial overnight precipitation with 33 g/L PEG solution, collect the precipitates and wash as above with ice-cold 25 g/L PEG-BSA solution. Centrifuge and collect the pellets; repeat the wash procedure. Resuspend the final pellet in 0.2 mL of Tris buffer for assay, as above. The pellets disperse more easily in PEG when BSA is

¹ Department of Laboratory Medicine, Sinai Hospital of Detroit, 6767 W. Outer Drive, Detroit, MI 48235.
² Ligand Assay Laboratory, Henry Ford Hospital, 2799 W. Grand Blvd., Detroit, MI 48202.
³ Nonstandard abbreviations: IC, immune complexes; PEG, polyethylene glycol; AHG, heat-aggregated gamma-globulin; BSA, bovine serum albumin; PBS, phosphate-buffered isotonic saline; and IRMA, immunoradiometric assay.

Received April 6, 1984; accepted May 29, 1984.
included in the solution. Occasionally one has to use a rod to disperse a pellet. The results described below were obtained with use of the longer extraction procedure, except for those assayed by the IRMA, which were obtained with the shorter extraction.

Absorbance nephelometry. Add 50 μL of the final suspension separately to 350 μL of test and 350 μL of blank assay solution (per liter: 6.3 g of PEG, 15 mmol of PBS, pH 7.4 ± 0.1). The test solution also contains 20 μL of anti-human IgG per milliliter, while the blank does not. Incubate for 30 min at room temperature and read the absorbance in the spectrophotometer. The difference in absorbance between the test and blank solutions for each sample is attributed to turbidity caused by the reaction of anti-IgG with IC.

IRMA. Add 25 μL of the final extract to 375 μL of assay solution (per liter: 25 g of PEG, 15 mmol of PBS, pH 7.4). The assay solution also contains, per milliliter, 20 μL of carrier anti-IgG and 10 μL of 125I-labeled anti-IgG. Incubate for 30 min at room temperature; add 0.6 mL of ice-cold assay buffer (without anti-IgG), shake, and let the samples stand overnight at 4 °C. The next morning, centrifuge, decant the supernate, and count the radioactivity of the pellets.

Calibrators in Tris buffer are not precipitated with PEG, but rather are added directly to the reaction mixture in the same volumes as the samples extracted from sera.

Standardization. Standardization for the spectrophotometric method has been described (2). In brief, dilute AHG with Tris buffer to give concentrations ranging between 12.5 and 400 mg/L. Absorbance readings for the calibration curve range between 0.02 and 0.6 A for the lowest and highest calibrators, respectively; concentrations greater than 400 mg/L are reported as >400.

For standardization of the IRMA, we generate assay curves from monomeric IgG calibrators of between 12.5 and 800 mg/L. For most experiments, we counted radioactivity until we accumulated between 30 000 and 40 000 counts for the lowest calibrator and between 120 000 and 140 000 counts for the highest: these represented about 20 and 75% of the total counts, respectively.

Results

Extraction methods. Table 1 summarizes the precision of the shortened extraction procedure for several serum samples containing either AHG or endogenous IC as assayed over a period of several weeks by the spectrophotometric method. The CV of about 10% agrees favorably with that of the longer extraction (2). The reproducibility is well within clinically useful limits for this type of assay.

The correlation between results by the longer (x) and the shorter (y) extractions was r = 0.93 (n = 35, y = 0.9x + 3.6). Four samples that measured >400 mg/L from both extraction procedures were not included in the calculation of regression line. The Spearman rank correlation for all 39 samples was ρ = 0.92. From the regression line, an upper limit for normal value of 45 mg/L obtained with the shorter extraction procedure corresponded to 46 mg/L by the longer extraction procedure (2).

Correlation between methods. The correlation between the spectrophotometric and C1q-binding assays (r = 0.86, n = 112) is shown in Figure 1. Samples exceeding 400 mg/L (open symbols) were not included in the statistical calculations. This degree of correlation is remarkably high for determinations of IC performed in different laboratories on the basis of different theoretical principles.

Correlation coefficients for all assays are shown in Table 2. There is good agreement between the anti-IgG and C1q-binding assays but poor agreement between the anti-IgG and Raji cell assays. Omitting from calculation the results for 10 samples that were discordant between the Raji cell assay and the other assays increased the correlation coefficients to r = 0.46 (n = 43, p < 0.005; Scripps), and r = 0.64 (n = 63, p < 0.001; SmithKline). The discrepant values are detailed in Table 3.

Sensitivity for detecting increases of IC. Table 4 indicates that the anti-IgG and Raji cell tests were significantly more sensitive for detecting increased concentrations of IC in patients' samples than was the C1q-binding test. Samples from patients with systemic lupus erythematosus, bacterial endocarditis, and rheumatoid arthritis—diseases associated with high concentrations of IC—more often had high test results when assayed by the anti-IgG test than by the C1q-binding test.

Calibration of IRMA. Figure 2 demonstrates the relationship between isotope binding and concentration when samples containing monomeric IgG, unextracted AHG, AHG extracted by PEG from control sera, and PEG-extracted endogenous IC were assayed by IRMA. A high degree of parallelism among all samples is apparent.

Discussion

Because IC precipitate from serum at PEG concentrations between 20 and 35 g/L, whereas monomeric IgG does not (6),
many investigators have used this reagent to separate IC from monomeric IgG. Early researchers measured total protein in PEG precipitates (7) or IgG by radial immunodiffusion (8). These methods suffer from poor specificity and precision. Although IC from PEG precipitates have been measured successfully by a reliable radioassay technique with protein A as the indicator (9), anti-IgG has several advantages over protein A: reactivity with a wider range of IgG subclasses, greater commercial availability, and precipitation formation, which lends itself to nephelometry as well as radioassay. As we have shown elsewhere (2), monomeric IgG does not interfere with the assay when an appropriate extraction precedes the assay, and high analytical recovery of AHG from serum is possible.

The present study shows a high linear correlation between the anti-IgG assay and the C1q-binding test, independent of the technique used to measure the binding of anti-IgG (Table 2). The correlation between the spectrophotometric method and the C1q-binding test (Figure 1) leaves little doubt that these tests are largely measuring the same variables. C1q-type tests suffer from poor recovery (10, 11), difficulty in standardization (10), and a dependency of reaction rate on the size of the IC aggregates (12), problems that can confound interlaboratory duplication of results (13). However, the similarity in regression lines between the C1q-binding test, performed in different laboratories, and the anti-IgG test (Figure 1) indicates that good interlaboratory agreement can be attained with the C1q-binding-type-

**Table 2. Correlation between Results of Anti-IgG Assays and Those of Other Assays for IC**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Spectrophotometric</th>
<th>Light scatter*</th>
<th>ANA</th>
<th>IMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1q-binding</td>
<td>r</td>
<td>0.86</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>—</td>
<td>0.82</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>112</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Raji cell</td>
<td>r</td>
<td>0.269*</td>
<td>0.90</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>46</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.176*</td>
<td>0.85</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>70</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Light scatter was performed with a Beckman ICS by adding 42 μL of antiserum according to the ICS protocol to 600 μL of buffer (polymer enhanced, 20–30 g/L PEG, PBS; Kallestad Laboratories, Austin, TX 78701) containing 42 μL of PEG extract from serum. Concentrations of IC were read from the instrument in IgG equivalents.

a Performed by SmithKline.
b Performed by Scripps.

**Table 3. Discordant Values between Assays**

<table>
<thead>
<tr>
<th>Anti-IgG</th>
<th>Raji cell</th>
<th>C1q-binding</th>
<th>AHG, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>158</td>
<td>900</td>
<td>11</td>
<td>SmithKline</td>
</tr>
<tr>
<td>105</td>
<td>500</td>
<td>19</td>
<td>SmithKline</td>
</tr>
<tr>
<td>166</td>
<td>500</td>
<td>20</td>
<td>SmithKline</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>8</td>
<td>SmithKline</td>
</tr>
<tr>
<td>34</td>
<td>874</td>
<td>7</td>
<td>SmithKline</td>
</tr>
<tr>
<td>138</td>
<td>500</td>
<td>13</td>
<td>SmithKline</td>
</tr>
<tr>
<td>110</td>
<td>1139</td>
<td>10</td>
<td>SmithKline</td>
</tr>
<tr>
<td>105</td>
<td>1996</td>
<td>5</td>
<td>Scripps</td>
</tr>
<tr>
<td>47</td>
<td>547</td>
<td>17</td>
<td>Scripps</td>
</tr>
<tr>
<td>136</td>
<td>1619</td>
<td>18</td>
<td>Scripps</td>
</tr>
</tbody>
</table>

*Antinuclear antibody titer 1:640. #ANA titer 1:320. $ANA titer unknown.

**Table 4. Sensitivity of IC Assays**

<table>
<thead>
<tr>
<th>Test</th>
<th>No. positive</th>
<th>No. negative</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>62</td>
<td>54</td>
<td>—</td>
</tr>
<tr>
<td>Raji cell</td>
<td>66</td>
<td>50</td>
<td>0.5 &lt; p &lt; 1.0</td>
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<tr>
<td>C1q-binding</td>
<td>30</td>
<td>85</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Patients with IC diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>28</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Raji cell</td>
<td>25</td>
<td>4</td>
<td>0.1 &lt; p &lt; 0.5</td>
</tr>
<tr>
<td>C1q-binding</td>
<td>15</td>
<td>14</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

*No. positive indicates those with results above the upper limit for normal (see footnote to Table 3).

*No. negative indicates those with results within normal limits.

*By chi-square test relative to anti-IgG spectrophotometric method.

Fig. 2. Relationship between binding of 125I-labeled anti-IgG and concentration of IgG in the sample

O, AHG in buffer without PEG extraction; $, monomeric IgG in buffer (mean of five runs); x, endogenous IC extracted by PEG from patient no. 1; D, IC from patient no. 2.

The horizontal bars represent the range of values for monomeric IgG (n = 5)

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tests. Technical advantages of the anti-IgG approach include stability of the anti-IgG molecule, greater commercial availability, ease of storage, and stability of the radiolabeled molecule.

The spectrophotometric assay can be performed with good precision by generating a standard curve from the average of three or four runs of freshly prepared AHG, and by using this curve for subsequent assays with the same lot of antisera. Because AHG preparations are not always stable upon storage, we recommend using this approach, rather than generating a new curve for each assay. For quality control, we extract three serum controls and assay these each day along with two unextracted calibrators. This, along with the shorter extraction, simplifies the procedure for the routine laboratory.

Different preparations of AHG show poor consistency. Besides, AHG often loses activity within two months of preparation, even when stored at \(-80^\circ\text{C}\), so that a stock preparation cannot be maintained for a long time. As a result, laboratory standardization of these assays is difficult and time-consuming. To circumvent this problem, we examined the possibility of substituting monomeric IgG for AHG to calibrate the anti-IgG type IC assay. There was good parallelism for the IRMA results between curves generated from AHG, monomeric IgG, and endogenous IC (Figure 2) when we used the 25 g/L PEG buffer for the assay. Similarly, parallelism of results was good for endogenous IC as assayed by kinetic light scatter with the Beckman ICS, when we used monomeric IgG for standardization (data not shown). The correlation data for IRMA and light scatter shown in Table 2 were obtained by using monomeric IgG for standardization. Although these data were generated from a limited number of samples, the highly significant linear and rank correlation coefficients, along with the good parallelism between AHG and monomeric IgG, indicate good potential for this calibration approach. Coupled with the use of controls prepared from serum pools containing low, medium, and high concentrations of endogenous IC, this approach would eliminate entirely the need to use AHG during routine assay and obviate the problems associated with it.

Alternative methods for detecting IgG in IC—IRMA and kinetic light scatter—offer two advantages over the spectrophotometric assay. First, they are less tedious to perform. When automated data processing is available, the IRMA method lends itself to processing many samples; the light-scatter technique, on the other hand, is convenient for assaying fewer samples in a single day. Second, because the final PEG precipitate resists solubilization in aqueous buffer, the high background turbidity may cause poor reproducibility of results for some samples with the spectrophotometer. Background turbidity, however, should not interfere with the measurement of rate of change of precipitin formation, or with the measurement of radioactivity. The correlation data (Table 2) indicate that these methods can provide results similar to those of the C1q-binding test and the anti-IgG spectrophotometric assay.

Although a high linear correlation was found between the C1q-binding and anti-IgG tests, the correlation between Raji cell and anti-IgG tests was poor (Table 2). If samples that showed discrepant results (Table 3) are omitted from the calculation, the correlation increases dramatically (r = 0.46, Scripps; r = 0.514, SmithKline). The very high IC concentrations (>10-fold normal) indicated by the Raji cell test in some patients disagree with both the C1q-binding (<twofold normal) and the anti-IgG tests (Table 3). One possible explanation for this discrepancy is that neither PEG nor C1q precipitate low-molecular-mass IC, so that these assays do not measure smaller complexes although the Raji cell assay does. Another explanation, which seems more plausible to us, involves interference by autoantibodies.

Interference from antinuclear antibodies and other autoantibodies causes false-positive results with Raji cell assays (3, 4, 14). Recently, antinuclear antibodies have been shown to react with phosphodiester groups in phospholipids, the amount of activity being unique for individual antibodies (15, 16). Other studies have demonstrated anti-phosphatidylinositol, anti-cardiolipin, and other anti-phospholipid antibodies in the sera of patients with systemic lupus erythematosus (17)—antibodies shown to be responsible for false-positive syphilis test often seen when lupus patients are tested with the cardiolipin-based VDRL. The mechanism responsible for interference with the Raji cell test may involve the binding of the antibodies to the phosphodiester in the Raji cell membrane so that labeled anti-IgG binds to IgG in antibodies rather than only to the IgG in IC. This mechanism would explain why the interference is not necessarily proportional to the concentration of antibody in the sample. Consistent with this hypothesis, we found that for all discordant samples for which the patient’s record included these results (7/10), antinuclear antibodies were increased (Table 3). We are currently investigating this hypothesis in more detail by measuring Raji cell activity in samples before and after treatment designed to remove monomeric antibodies.

Some samples from patients with a clinical picture of IC disease (especially systemic lupus erythematosus) show a dissociation between C1q-binding and Raji cell assay results (1, 18). In such cases (e.g., Table 3) the clinician is faced with a dilemma. If the C1q-binding test is correct, the concentration of IC is normal, contrary to the clinical picture, whereas if the Raji cell assay is correct, the IC is extremely high. Our data suggest that the C1q-binding test contributes to this problem by poor sensitivity for detecting IC (Table 4), whereas the Raji cell value may be inflated.

In most studies examining the clinical usefulness of distinguishing between complement-binding activity of IC and bound complement activity, the Raji cell test has been used to measure bound complement. If the above hypothesis regarding interferences with the Raji cell test is correct, these studies may have to be re-evaluated with use of a more specific indicator of bound complement activity. We question whether it is economically justified or clinically useful to continue to perform both tests. The anti-IgG test described here may be helpful in answering this question.

Because of the large and heterogeneous nature of the molecules, most circulating IC is assumed to contain all three major immunoglobulin classes. IgG, however, the major immunoglobulin in serum, is expected to be the predominant immunoglobulin class in IC. A sensitive, reliable test for IgG in circulating IC that is straightforward for use in routine clinical laboratories could thus serve as an initial screening test for IC. The approach we describe here should provide the basic diagnostic information about IC for most patients. In rare instances, diseases may be associated with IC containing immunoglobulins that are primarily not IgG (19, 20); in other cases, more specific information may be deemed clinically necessary. In such conditions, supplemental specialized testing specific for other immunoglobulin classes or particular antigens in IC would be necessary.

References


