Erroneous Results with Routine Laboratory Testing for Immunoglobulins Due to Interference from Circulating Immune Complexes in a Case of Hyperviscosity Syndrome Associated with Autoimmune Disease

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A patient with the hyperviscosity syndrome exhibited very high concentrations of intermediate to small circulating immune complexes (CIC), involving 40–50% of the IgG present, with IgG rheumatoid factor activity. We demonstrate that precipitation of CIC by polyethylene glycol in the reaction mixture caused interference with nephelometric methods for measuring IgM and IgA, and that failure of immunoglobulins to migrate, owing to molecular interactions, caused interference with radial immunodiffusion methods. Semiquantitative values for immunoglobulins were difficult to interpret on immunoelectrophoresis. As a result, IgM and IgA could only be quantitatively estimated by an end-point nephelometric approach that included a serum blank. Immunoelectrophoresis indicated that a large proportion of the immunoglobulins behaved as aggregates. Immunofixation electrophoresis did not reveal the presence of aggregates. The polyethylene glycol–IgG test provided an accurate assessment of the CIC concentration; the Raji cell and C1q-binding assays did not. Evidently, special techniques may be necessary for accurate determination of immunoglobulin concentrations when CIC concentrations are very high.

Additional Keyphrases: polyethylene glycol–IgG CIC assay · radial immunodiffusion · nephelometry · immunoelectrophoresis · IgG rheumatoid factor · gel-filtration chromatography

Usually, hyperviscosity syndrome is associated with increased concentrations of monoclonal IgM (Waldenström’s macroglobulinemia) or multiple myeloma. Circulating immune complexes (CIC) have also been shown to cause this syndrome (1). Despite the paucity of cases, the hyperviscosity syndrome associated with CIC has been well reviewed (2–4). Symptoms of hyperviscosity include circulatory overload (congestive heart failure), bleeding diathesis, somnolence, decreased vision with dilated retinal veins, anorexia, fatigue, and malaise, when the relative (to water) viscosity reaches 6 to 7 (5), although patients with vascular disease may exhibit symptoms at lower viscosities (3). Patients with hyperviscosity syndrome ascribable to CIC commonly have lymphadenopathy and liver involvement, and are positive for rheumatoid factor (RF). Plasmapheresis is usually effective in relieving the symptoms, although patients may also require treatment with steroids (2).

The RF, which binds to IgG to form CIC, may be IgG or IgM, or both. Usually the complexes are of the intermediate size 9–17S, as first described by Kunkel et al. (6), but complexes as large as 45S have been described (1). Usually, the immunoglobulin components are polyclonal (4), but one case has been described with monoclonal IgA RF involvement that was unrelated to multiple myeloma or lymphoma (7).

Here we describe the adverse effects of high CIC concentrations on some laboratory procedures for identifying and measuring immunoglobulins. The CIC concentration in this patient’s serum was 26 g/L (approximately 45% of the IgG). This high CIC concentration was precipitable with polyethylene glycol (PEG) in concentrations of 25 g/L, before antisera was added, causing an interference with methods for measuring immunoglobulins by rate nephelometry, because in these methods 20 to 40 g of PEG per liter is commonly used in the assay medium to facilitate the antibody–antigen reaction. The serum also showed erroneous results for immunoglobulins when assayed by radial immunodiffusion (RID), and caused difficulty in the interpretation of immunoelectrophoresis.

Case Report

A 68-year-old woman entered the hospital because of symptoms of hyperviscosity syndrome, including circulatory overload, lassitude, dizziness, gastric bleeding, and decreased vision with dilated retinal veins. Pertinent clinical and hematological tests indicated non-iron-deficiency anemia, slightly increased gamma-glutamyltransferase, creatinine of 20 mg/L (normal 7–18), urea nitrogen 360 mg/L (normal 100–200), total protein 98 g/L (normal 60–80 g/L), albumin 26 g/L, and relative (to water) viscosity 4.04 (normal 1.4–1.8). Immunological tests showed the complement C3 concentration to be 1500 mg/L (normal 700–1780), C4 620 mg/L (normal 63–447), IgA 2700 mg/L (normal 500–2000), IgM 1920 mg/L (normal 500–1500), IgG 59.3 g/L (normal 5–15), antinuclear antibody positive at 1:160, RF positive at 1:1280, and a positive direct Coombs test. Tests for CIC indicated 2.093 g/L by the Raji cell (normal <0.05), 99% by the C1q-liquid phase (normal <13), and 26 g/L by the polyethylene glycol precipitation–IgG test (PEG–IgG) (normal <0.046). Results of immunofixation electrophoresis (IFE) and immunoelectrophoresis are shown in the Results section.

Biopsy of lymph nodes indicated diffuse lymphadenopathy in the axillary, cervical, and inguinal areas. Some changes were suggestive of Castleman’s disease, but the overall picture was that of nonspecific severe lymphadenitis.

Materials and Methods

Electrophoresis. We performed IFE and immunoelectrophoresis, using the Panagel® system (Princeton Separation Products Inc., Freehold, NJ 07728). Antisera against IgG, IgA, IgM, and lambda were obtained from Meloy Labs,
Springfield, VA 22151. Antiserum to kappa was obtained from Tago Inc., Burlingame, CA 94010.

Measurement of immunoglobulins. For rate nephelometry we used the "Immunochemistry System II" (Beckman Instruments, Fullerton, CA 92634). For end-point turbidimetry we used the same reagents as for rate nephelometry, according to the following protocol: Prepare a calibration curve by serial dilution of the calibrators. Add a 16-fold dilution (with isotonic saline) of calibrators, control, and patients' serum. Add 20 µL of the diluted samples to 0.6 mL of buffer, in duplicate. Add 42 µL of antiserum to one of the duplicates (test) and 42 µL of saline to the other (blank). Incubate for 2 h at room temperature. Measure the absorbance at 340 nm in a double-beam spectrophotometer (Beckman 25K), with the blank in the reference cell and the test in the sample cell. Construct a standard curve by plotting the results for the calibrators, and estimate the concentrations in the patients' samples and controls from this standard curve. For radial immunodiffusion (RID) we used plates from Kallestad Laboratories, Austin, TX 78701, and the Mancini–Heremans end-point technique, with a 48-h diffusion period.

Circulating immune complexes were measured by the Raji cell assay, the C1q-binding assay, and the PEG–IgG assay, as previously described (8). In the last technique, the CIC are precipitated with 25 g/L PEG, final concentration; after washing, the precipitate is assayed for IgG and IgM by a modification of the rate-nephelometric method for measuring immunoglobulins.

Gel-filtration chromatography was performed in the cold as previously described (9). We used a 2.5 × 50 mm glass column, fully packed with "BioGel A" 1.5-mm resin (Bio-Rad Laboratories, Richmond, CA 94804). The eluate in tubes 10–20 was pooled, as was that in tubes 21–30, and 31–40 to give three fractions (I, II, and III, respectively). Each fraction was concentrated at 4 °C to 1 mL in "Minicon B-12" concentrators (Amicon Corp., Danvers, MA 01923).

Separation of immunoglobulin classes for RF testing. We separated IgG from IgM and IgA, for RF testing, with a quaternary aminosil-Sephadex A-50 ion-exchange column (10), which is supplied as a kit (Isolab Inc., Akron, OH 44321). IgG is eluted with the wash solution, and IgM and IgA with a high-salt solution. RF activity in each fraction was determined with a latex-agglutination kit ("Rapi/Tex"; Behring Diagnostics, La Jolla, CA 92037).

Results

Electrophoresis. The results of immunoelectrophoresis can be seen in Figure 1. Immunoglobulin G appears to be slightly increased, IgA and kappa appear increased. Lambda appears normal, while the amount of IgM appears to be decreased. A boat-shaped precipitin line is seen near the center of each strip. This precipitin line represents a high CIC concentration.

Results by IFE are seen in Figure 2. There is a restriction in the gamma region at the cathodal end. Although initial immunofixation with a 36-fold dilution of antisera mistakenly suggested that this restriction might be monoclonal IgG-κ (Figure 2, above), fixation with various dilutions (Figure 2, below) indicated a polyclonal restriction containing IgG, IgM, and IgA. CIC cannot be identified by IFE.

Immunoglobulin concentrations. Immunoglobulin concentrations (g/L) as measured by rate nephelometry were IgA, 2.70; IgM, 1.95; and IgG, 59.30. Corresponding concentrations by RID were 1.04, <0.03, and 51.60 g/L.

Because of the discrepancy between IgM and IgA values as measured by rate nephelometry and RID, we examined the change in light scatter with time after the sample was added to the reaction buffer at the 36-fold dilution of serum customarily used for assaying IgM and IgA with the rate nephelometer, before the antiserum is added. Table 1 indicates that at a 36-fold dilution, after an initial high reading, the light scatter decreased for about 5 min, then increased to very high values for at least 2 h. At a 216-fold dilution, the light scatter increased gradually for as long as 2 h, similar to the changes seen for a normal specimen at a 36-fold dilution. At a 1396-fold dilution, which was necessary to measure the high IgG concentration in this sample, no anomalous pattern of light scatter was seen.

Better to evaluate the concentrations of IgM and IgA, we measured them by a turbidimetric end-point technique;
concentrations of IgA and IgM were 3.04 and 7.20 g/L, respectively.

Gel-filtration chromatography (Figure 3) indicated two components of IgG. A component consisting of aggregates of intermediate size (presumably dimers and trimers) was eluted in tubes 20 to 30, and a monomeric component between tubes 31 to 40, peaking near tube 35. As indicated in Figure 3, precipitation of the eluate with PEG indicated that the aggregated component was most concentrated in or near tube 26. We estimate this component to account for 40 to 50% of the IgG. Although a large portion of the IgM was eluted in tubes 20 to 25, presumably as tetramers and trimers, it does not seem to be associated with the IgG aggregates, nor was it possible to precipitate significant amounts of IgM with PEG.

RF activity was determined in whole serum and in the pooled eluates obtained from gel-filtration chromatography before and after separation of the immunoglobulin classes by ion-exchange chromatography. The results (Table 2) indicate that the RF activity was associated with IgG rather than with IgM or IgA.

Discussion

In this case of hyperviscosity syndrome caused by IgG–RF, approximately 40 to 50% of the IgG was circulating as CIC. We show how such high CIC concentrations can interfere with methods commonly used for measuring immunoglobulins. Two areas of interference are pronounced:

1. In quantification of IgA and IgM, interference was seen with both rate-nephelometric and RID techniques. The end-point turbidimetric method probably gave more nearly correct results for those concentrations, because the sum of the immunoglobulins measured by it (IgA = 3.0 g/L, IgM = 7.2 g/L) along with the sum of the IgG (59.3 g/L) and the albumin (26 g/L) adds up to nearly the amount of total protein (98 g/L). Interference with nephelometry presumably is ascribable to the precipitation of aggregates in the reaction solution by the PEG that is used to facilitate the antibody–antigen reaction. In most methods 25–40 g/L is used for this purpose. Because of the higher concentrations of IgG in serum, the large (216-fold) dilution eliminated interference with IgG measurement (Table 1). We expected that this precipitation would cause falsely high immunoglobulin values. Surprisingly, with the rate-nephelometric

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**Table 1. Light Scatter from Precipitation of CIC in the Reaction Buffer**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Patient’s serum</th>
<th>Normal serum</th>
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<tr>
<td></td>
<td>1:36</td>
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<tr>
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<td>1:36</td>
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<td>&gt;800</td>
<td>47</td>
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**Fig. 3. Results of gel-filtration chromatography**

Closed circles represent the elution of IgG; ×, IgM; open circles PEG–IgG. WV indicates the end of the void volume; 19S, the peak of pentameric IgM; 7S, IgG. The left-hand ordinate indicates the concentration of IgG, the right-hand ordinate the concentrations of PEG–IgG and IgM. Tube numbers are shown on the abscissa.
method, the IgA concentration (2.7 g/L) was nearly correct, and the IgM concentration appeared to be too low. These findings were probably due to the decrease in light scatter observed during the first 5 min (Table 1). It is clear from Table 1 that, after the first 5 min, the increase in light scatter would cause positive interference with measurement of immunoglobulins by end-point or rate-nephelometric and turbidimetric methods that involve longer incubation intervals without individual separate sample blanking (such as in centrifugal analyzers). Because of lower concentrations, measurement of complement should be similarly affected.

The apparently incorrectly low values for IgA and IgM by RID are more difficult to explain. We were unable to precipitate high amounts of IgM and IgA with PEG (results not shown), neither IgA or IgM showed RF (Table 2), and the IgM did not seem to be associated with the aggregates upon gel-filtration chromatography (Figure 3). Therefore, although decreased migration of immunoglobulin aggregates is well described, the IgA and IgM did not seem to be part of the aggregates, and we did not expect a slower migration than normal. Apparently, there was a loose molecular association between these immunoglobulins and the IgG aggregates, sufficient to inhibit migration.

2. Although IgG and IgM concentrations were shown to be very high by end-point turbidimetry, values for IgG and IgM by immunoelectrophoresis were difficult to interpret (Figure 1); IgG appeared only slightly increased, while IgM actually appeared decreased. A very large precipitin zone was near the middle of each gel, consistent with a very high CIC concentration. It was this that led us to suspect CIC as the cause of hyperviscosity. Apparently, as with RID, the IgM was unable to migrate free of the aggregates. IFE (Figure 2) does not reveal the presence of CIC. Although IFE has clear advantages over immunoelectrophoresis for identifying multiple monoclonal gammapathies, and for identifying monoclonal IgM associated with lymphoma, it was at a disadvantage in the case described here.

Finally, with regard to the methods for measuring CIC, concentrations measured by the PEG-IgG method were congruent with the clinical symptoms and the results of column chromatography. We have found that there is generally a good semiquantitative relationship between concentrations of CIC as measured by the PEG-IgG method and that identified by gel-filtration chromatography (unpublished results), and this relationship seems to hold over a wide range of aggregate sizes. In this type of hyperviscosity with a positive RF, but no M-components present, it appears that CIC, as measured by the relatively simple PEG-IgG assay, suffices to explain the hyperviscosity. On the other hand, although results by the C1q-binding and the PEG-IgG methods correlate well, the C1q-binding assay falls off in the high range, and cannot distinguish between high and very high CIC concentrations (11, 12). The Raji cell result (2.093 g/L) did not accurately reflect the concentration of aggregates.

The results presented here are not in agreement with those of some other studies indicating that only CIC of a large size (>25S) are precipitated by PEG (13, 14). The precipitation of smaller CIC with 25 g/L PEG is not peculiar to the very high concentrations of CIC found in this patient. With our method, we have been able to precipitate smaller CIC from several sera, including some with CIC concentrations as low as 400 mg/L (unpublished observations).

References