A hemolytic method for the measurement of nephritic factor

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Abstract

The absence of a simple and widely applicable test for the measurement of NF activity has hampered the accumulation of evidence bearing on its nephritogenicity. The extensive modification of a screening test for this autoantibody, reported here, has increased the range and precision of the test and made it less laborious. C3b deposited on sheep E by the reaction of NF with NHS forms a C5 convertase which, with addition of rat EDTA serum, leads to hemolysis of the cells proportionate under the right conditions to the concentration of NF in the reaction mixture. The calibration line is straight or slightly concave and passes through the origin. The method detects the activity of both the NF of the amplification loop, NFa, found in MPGN type II, and the NF of the terminal pathway, NFt, found in MPGN types I and III. Interday coefficients of variation ranged from 6.6% to 13.5% and intraday from 7.0% to 12.6%. Although serum C3 levels can be markedly depressed when NF levels are high, C3 levels and NF activity generally correlate poorly. The C3 level could be low and NF absent or, occasionally, NF present with the C3 level normal. NF activity was absent from the stored serum of patients with active SLE, AGN or with an IgA nephropathy.

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1. Introduction

NF is an autoantibody directed against activated factor B, a constituent of the C3 convertase, C3b,Bb. Native C3b,Bb rapidly decays but its half life is increased many fold when it is in the form, NF,C3b, Bb. When this stabilized convertase is abundant, as in membranoproliferative glomerulonephritis or partial lipodystrophy, the circulation can be depleted of C3. The C3 is converted to C3b which is in turn rapidly degraded to C3c and C3dg.

C3b,Bb can be stabilized by either of two NFs. One, known as the NF of the terminal pathway, NFt, requires properdin for its formation and the stabilized C3b,Bb activates not only C3 but also C5 and other terminal components (Mollnes et al., 1986; Clardy et al., 1989;
Tanuma et al., 1990). This form appears to be confined to MPGN types I and III. The NF of the amplification loop, NFa, found in partial lipodystrophy and MPGN type II, is properdin independent and does not activate terminal components. These differences in reactivity of the convertase, dependent on the stabilizing nephritic factor, are reflected in differences in the complement profiles they produce in vivo (Varade et al., 1990).

Observations in experimental animals as well as on rare conditions in man provide indirect evidence that NF is nephritogenic. Several patients have developed MPGN resembling type II under circumstances in which C3b,Bb circulates because of uncontrolled activation of the amplification loop due to non-functional factor H. The non-function has been the result of either homozygous factor H deficiency (Levy et al., 1986; Lopez-Larrea et al., 1987; Dragon-Durey et al., 2004) or of a circulating inhibitor of the binding of factor H to C3b (Meri et al., 1992; Jokiranta et al., 1999). In animals, uncontrolled activation due to factor H deficiency also produces a disease resembling MPGN (Jansen et al., 1998; Pickering et al., 2002). The common denominator in the generation of the nephritis in these conditions is thought to be uncontrolled activation of C3 (Appel et al., 2005). In fact, when C3 activation is prevented in factor H deficient animals by inbred absence of factor B, nephritis does not develop (Pickering et al., 2002). From the above, it may be reasoned that the uncontrolled C3 activation resulting from the rendering of factor H ineffective by NF would also predispose to nephritis.

The failure of clinicians observing patients with MPGN type II over the past several decades to assemble evidence that NF is nephritogenic is due in part to the lack of a suitable method for its measurement. A method employing radial immunodiffusion developed in this laboratory shortly after the recognition of NF (Vallota et al., 1971) was not highly sensitive and was not widely used because it required an infrequently produced antibody to a unique epitope on C3. The method employing crossed immunoelectrophoresis is semi-quantitative and insensitive to low concentrations (López-Trascasa et al., 1987). Other methods requiring purified complement components have seen only limited use (Ohi et al., 1992; Seino et al., 1993). As a result, assessment of the role of NF in pathogenesis has been largely based on an assumed negative correlation between the levels of NF and C3. However, several factors in addition to NF can modulate the C3 level making this correlation unsuitable as a basis for judging NF nephritogenicity.

The method to be described is a modification of a screening test for NF developed by Rother (1982) based on the hemolysis of sheep E by terminal components. The amount of hemolysis is dependent on the amount of C3b deposited on the cells, in turn dependent on the amount of convertase stabilized by NF. The reaction occurs in two steps. First, NF deposits C3b on the cells at 30 °C. Because a membrane attack complex formed with human C5 will not lyse sheep E, rat EDTA serum is added in the second step to provide an effective C5 and lysis occurs during incubation at 37 °C. The modification here reported is superior to the screening method (Rother, 1982) in that it is more sensitive, more quantitative and eliminates the extensive washing of the sheep E which was a part of the original method.

2. Materials and methods

2.1. Buffer diluents

VBS containing EGTA, 16 mM; MgCl2, 4.8 mM; and gelatin, 0.3% (GVB EGTA Mg). VBS containing EDTA, 10 mM; and gelatin, 0.3% (GVB EDTA). The diluent for serum unknowns, blanks and calibrator is 10% melibiose containing 0.75% HSA.

Before use, sheep E (Colorado Serum Co., Denver, CO) are washed twice in GVB EGTA Mg and suspended in that buffer in a concentration of 1.34×10⁹/ml. When one volume of the suspension is lysed by adding 14 volumes of 0.15% anhydrous Na₂CO₃, the OD at a 1 cm light path and a wavelength of 545 nM should approximate 0.945. Cells which have been stored at 4 °C as long as 13 days in the original anticoagulant (Alsevers) or after washing have given satisfactory results.

2.2. Sera

NHS was obtained from the blood of healthy adults. The blood was allowed to clot overnight at 4 °C and the serum aliquoted and stored at −80 °C. Sera from many healthy adults is non-reactive or minimally reactive in the method. Before large volumes are donated, potential donors should be screened for the reactivity of their serum. Rat serum (Pel-Freez Biologicals, Rogers, AK) was aliquoted and stored at −80 °C. The serum from patients had been stored for five or more years at −80 °C.

2.3. Patients

The criteria for distinguishing the types of MPGN and for the diagnosis of AGN and of the nephritis of
SLE were given previously (Braun et al., 1999; West and McAdams, 1978a,b). NF activity was measured on specimens from 38 normal subjects, 13 patients with MPGN type I, 18 with type II, 19 with type III, seven with AGN, nine with active SLE and 10 with an IgA nephropathy. In all specimens except those from normal subjects, from patients with an IgA nephropathy and from a few with MPGN type II, the serum C3 concentration was low. Specimens from patients with MPGN were obtained at varying times in the course of the disease while those from patients with the other diseases were obtained shortly after onset or after relapse. Because the serum specimens had been stored for long periods, the need to obtain consent for their use was waived by the Institutional Review Board.

2.4. Measurement of NF

NHS and the suspension of washed sheep E in GVB EGTA Mg are cooled to 0 °C and 45 volumes of NHS and 55 volumes of the cells are gently mixed in a pre-cooled tube. One ml aliquots of this suspension are distributed to pre-cooled 5 ml plastic tubes which are then transferred to a room temperature water bath and frequently agitated to speed warming. After 6 min at room temperature, they are immersed in ice slush for 6 min with frequent agitation to hasten cooling. The contents are then pooled in a pre-cooled tube and held in ice slush for 40 min. During this period, 40 µl of dilutions of unknown serum, blanks and calibrator in melibiose-HSA are placed in pre-cooled 1.5 ml microcentrifuge tubes. At the end of the 40 min incubation, 200 µl of the serum-cell suspension are carefully added to each tube to overlie the sample in the bottom. When pipetting is complete, all tubes in a rack are mixed at the same time by repeatedly inverting and jarring the rack for approximately 30 s. They are then placed for 20 min in a 30 °C water bath. Agitation is not necessary in this bath. After cooling for 6 min in ice slush, a pre-cooled mixture of 4 parts of GVB EDTA and one part rat serum are added to each tube in a volume of 0.4 ml. Again, mixing is delayed until pipetting is complete and is by inversion and jarring. After incubation for 90 min at 37 °C without agitation, they are cooled, gently mixed by inversion and centrifuged. A volume of 0.1 ml of each supernatant is pipetted into flat bottomed wells in a 96 well polystyrene assay plate and the OD read in a Tecan Spectra at 412 nM with a background of 620 nM.

A 1/160 dilution in 10% melibiose-HSA of a pool of NHS served as a blank. The blank was run in duplicate or triplicate.

2.5. Dilution of unknowns

Although NF activity and C3 levels are not highly correlated (see below), the C3 level can act as a guide in selecting a dilution for an unknown serum. Suggested dilutions are given in Table 1. Because NF activity levels can vary widely at a given C3 concentration, an OD minus OD of the blank (Corrected OD) within range of the calibrators is more likely if two serial dilutions are used.

2.6. Calculation

The plot of the corrected OD determined for serial dilutions of serum containing NF by the above method versus the volume of serum in the sample, is slightly curvilinear becoming linear as it approaches the origin (Fig. 1). The dilution of serum at which the line curves only slightly or becomes straight is assigned a NF activity of 1000 arbitrary units/ml and this serum dilution together with a second serial dilution are used as calibrators. For the serum used as calibrator in the present study, these dilutions were 1/160 and 1/320. With NF activity in arbitrary units plotted on the abscissa from zero to 1000 and the corrected OD on the ordinate, a calibration line is drawn through the corrected ODs of the two calibrators and through the origin. In calculating the NF activity of an unknown, the NF activity of its dilution is first determined by applying the corrected OD to the calibration line. This value is then corrected for the dilution of the sample by multiplying that dilution by the reciprocal of the dilution of the calibrator assigned a value of 1000 units/ml. As an example, for an unknown serum diluted 1/320 with a corrected OD corresponding to 273 units/ml on the

<p>| Table 1 |
|---|---|
| Suggested dilutions of unknown sera based on their C3 concentrations |</p>
<table>
<thead>
<tr>
<th>C3 Mg/dl</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;25</td>
<td>1/10</td>
</tr>
<tr>
<td>24–15</td>
<td>1/20 and 1/40</td>
</tr>
<tr>
<td>14–9</td>
<td>1/80 and 1/160</td>
</tr>
<tr>
<td>8–5</td>
<td>1/160 and 1/320</td>
</tr>
<tr>
<td>&lt;5</td>
<td>1/320 and 1/640</td>
</tr>
</tbody>
</table>

a Assumes the lower level of the normal range for C3 concentration is 85 mg/dl.
calibration line, the NF activity would be 273 times 320 divided by 160 or 546 units/ml.

3. Results

3.1. Reproducibility

Coefficients of variation are given in Table 2. For ten measurements of NF activity in a day (intraday), the C.V. of the OD before subtracting the blank (uncorrected OD) varied from 1.5 to 4.2%. After subtracting the relatively high blank, the C.V. for NF activity increased to a range of 7.0–12.6% (mean, 9.7%). Interday variation was slightly greater, ranging from 6.6 to 12.6% (mean, 10.9%) for the uncorrected OD and from 7.4 to 13.5% (mean 11.5%) for NF activity.

Application to serum from normal subjects: When the method was applied to normal serum, the corrected OD for most specimens ranged from slightly above to slightly below zero (Fig. 1). Several specimens, however, had corrected ODs suggesting considerable NF activity. The serum C3 levels in these specimens were normal. Their NF activity was found to differ from that of “true” NF in that the corrected ODs of serial dilutions plotted versus the amount of serum in the reaction mixture were non-linear and did not intersect the origin. This difference, shown in Fig. 2, can be used to distinguish “true” from “false” NF activity.

Application to serum from patients with other nephritides: Fig. 1 shows a scattergram of the corrected ODs obtained when aged serum from patients with HSP-IgA nephropathy, SLE and AGN are tested. The mean and range of the C3 levels in these patients were as follows: HSP-IgA, mean 122 mg/dl, range, 105–204; SLE, mean 39 mg/dl, range 25–54; AGN, mean, 16 mg/dl, range 12–24.

3.2. NF stability

There was no evidence that NF lost its activity on prolonged storage at 80 °C or by repeated freezing and thawing. NF activity in dilutions of serum as great as 1/640 was not diminished by five cycles of freezing and thawing. This study, carried out over a period of several weeks, employed serum specimens from patients with MPGN type II as well as type III.

3.3. Correlating NF activity with the C3 level

The results of measurement of NF activity on stored serum specimens from hypocomplementemic patients with MPGN types I, II, and III, are shown in Fig. 3. The values are plotted against the C3 level. In all three types there is considerable scatter. In types I and III, there were a number of specimens with a low C3 level in which NF was absent. Also to be noted are the high

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>n</th>
<th>Serum dilution</th>
<th>NF activity mean units/ml</th>
<th>C.V. of uncorrected OD percent</th>
<th>C.V. of units/ml percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>III</td>
<td>10 1/160</td>
<td>446</td>
<td>1.5</td>
<td>8.5</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>10 1/40</td>
<td>170</td>
<td>2.1</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>10 1/40</td>
<td>167</td>
<td>4.2</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>10 1/40</td>
<td>147</td>
<td>2.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Interday</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>III</td>
<td>8 1/80</td>
<td>214</td>
<td>12.6</td>
<td>12.3</td>
</tr>
<tr>
<td>6</td>
<td>III</td>
<td>10 1/160</td>
<td>609</td>
<td>11.3</td>
<td>13.5</td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>9 1/30</td>
<td>141</td>
<td>6.6</td>
<td>7.4</td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>10 1/10</td>
<td>36</td>
<td>12.3</td>
<td>13.0</td>
</tr>
</tbody>
</table>

n — number of measurements.
c.v. — coefficient of variation.
levels in a number of specimens from patients with type III; six specimens were above 500 units/ml whereas only one specimen from the patients with the other two types was in this range.

The method is as applicable to NF$_t$ as to NF$_a$. Although a number of approaches were tried, no simple way to distinguish NF$_a$ from NF$_t$ was found.

4. Discussion

The principle of this method of NF measurement was taken from a screening method developed by (Rother, 1982). Her method calibrated in 50% hemolytic units was found by others to be superior to that employing crossed immunoelectrophoresis (López-Trascasa et al.,...
1987). It was developed using a single serum specimen with a high NF concentration and was designed to be especially applicable to high concentrations. The sensitivity of the present method is much greater. In addition, it eliminates the three washes of sheep E between the two incubations, a part of the Rother method.

The order of events and the temperature of the reactants at the time of mixing of the sheep E with the NHS target were found by trial and error to be critical for the reliability and reproducibility of the method. A problem encountered early in the development of the method was that the hemolysis produced by low concentrations of NF was relatively low resulting in a calibration curve convex to the abscissa rather than straight or slightly concave toward the abscissa. Another defect was that the corrected OD of a calibrator placed in one of the first tubes in a run was higher than that of the same calibrator in a tube at the end of the run. One change which partially corrected these problems was to mix sheep E and NHS when cold, then rapidly bring the mixture to room temperature for a short time and finally rapidly chill it to 0 °C and maintain it at that temperature for 40 min. Another was to raise the concentration of gelatin in the GVB EGTA Mg buffer from 0.1% to 0.2%.

A high degree of variability of replicates was remedied by several modifications. The variability was found in part to be produced by vortexing the sheep E–NHS mixture. The mechanical hemolysis so produced was eliminated by mixing the tubes by inversion. In addition, at 0 °C, NF was apparently effective in the cell–NHS mixture in depositing some C3b on the sheep E. As a consequence, variability in the mixing of fluid and solid phases while the reaction mixture was at this temperature gave variable final results. This was successfully countered by diluting the sample in 10% melibiose, depositing it at the bottom of the microcentrifuge tube and carefully overlaying it with the cell–serum mixture. The reverse order of addition, pipetting the sample underneath the cell serum mixture, is equally effective.

Finally, interday variability was found to be greatly reduced by a further increase in the gelatin concentration to 0.3%. It is not known why this was effective.

The ratio of NHS to suspended washed sheep cells of 45:55 and the concentration of sheep cells of $1.34 \times 10^9$/ml maximized the corrected OD of the calibrator.

The reason NF activity is inhibited when serum from some normal individuals was used as the target serum is not clear. Mixing experiments gave evidence that an inhibitor was involved rather than the absence of a factor. The inhibition is reminiscent of that found in the serum of patients with active SLE (Waldo et al., 1985). No attempt was made to identify the “false” NF found in the serum of a minority of apparently normal persons. It should be tested for in specimens with definite NF activity in the face of a normal C3 level.

A lack of correlation between C3 levels and NF activity, as shown in Fig. 3, was also observed in a previous study employing a different method for NF measurement (Vallota et al., 1972). Three factors could account for the lack of correlation. First, depression of C3 levels in the face of little or no NF activity could be a consequence of negative feedback on C3 synthesis produced by circulating C3 breakdown products (Charlesworth et al., 1974). Second, in MPGN type I, classical pathway activation could depress C3 levels in the absence of NF or augment the depression produced by NF. That classical pathway activation is occurring in this type is evidenced by both the complement profile and the composition of the glomerular deposits (Jackson et al., 1987; Varade et al., 1990). Finally, contributing to the lack of correlation could be the increased C3 synthesis which accompanies an acute phase reaction. In this event, C3 levels could be relatively high for the level of NF as seen in a few specimens from the patients with type II.

Whereas there is strong evidence that NF is nephritogenic, it can be tolerated for long periods even at high concentrations by some subjects while in others it produces rapidly progressive disease. Its nephritogenicity may in part depend on the availability of C3 and factor B. In sorting out these interrelationships, an accurate method for the measurement of NF is essential. The method will also be useful in judging the effectiveness of therapeutic modalities in eliminating this autoantibody.

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References


