Performance of a New Rate-Nephelometric Assay for Rheumatoid Factor, and Its Correlation with Tube-Titer Results for Human Sera and Synovial Fluid

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We evaluated a rate-nephelometric assay for rheumatoid factor (RF), as developed for use with the Beckman Immunochemistry System (ICS). Within-run precision (CV) for low-, mid-, and high-dilution samples (60–400 kilo-int. units/L) was 3.5, 1.5, and 1.6%; between-run precision was 3.4%. Analytical linearity was excellent. Biological interference resulted in some degree of nonlinearity in more than 70% of the patients' samples tested. Sensitivities were 96.5 and 94.1% and specificities were 98.5 and 95.3% for the ICS RF and Wampole slide methods, respectively, for a clinically defined population of 170 patients. Results for 100 ICS RF-positive samples correlated well with concomitantly measured Calbiochem-Behring tube-titers. Weekly measurement of Calbiochem-Behring, Hyland Diagnostics, and ICL Scientific tube-titer values for RF along with the ICS RF values on samples from the same patients indicated stable ICS RF values but showed at least ±1 tube dilution variances both within and between the tube-titer methods. Therefore, it may not be appropriate to compare a precise new method with a relatively imprecise comparison method. Treatment of RF-positive samples with dithiothreitol, which disrupts the pentameric character of immunoglobulin M, rendered the samples negative by repeat ICS RF assay and confirmed the method's specificity for pentameric immunoglobulin M–RF. Serum RF concentration as determined by ICS paralleled changes in clinical symptoms in a patient treated with both effective and relatively ineffective regimens, which suggests a useful role for the assay in monitoring efficacy of clinical treatment.

Additional Keyphrases: immunoglobulins • nonlinearity within a patient's samples • variation, source of • rheumatoid arthritis

The presence of circulating pentameric IgM anti-IgG antibodies, termed rheumatoid factor (RF), is a common serological finding in rheumatoid arthritis (1). In earlier procedures IgM–RF was roughly estimated visually by the ability of patients' sera to agglutinate various IgG-coated particles, e.g., erythrocytes (2), latex particles (3), or bentonite (4). The highly subjective nature of these measurements, their generally accepted precision of ±1 tube dilution, and the lack of calibration against a fixed standard have made it difficult to relate titers from one method to those from another (5–7).

Recently, enzyme-linked immunosorbent assay and rate-nephelometric methods have been described, which overcome some of the shortcomings of titer methods by providing more objective measurement of the IgM–RF (5, 8). However, this technique requires multiple incubation and washing steps, and several standards are needed with each assay (9). A rate-nephelometric method that requires only a paired single-point calibrator assay and relatively little technologist time could therefore provide a major advance in the ability to monitor changes in RF during treatment and disease progression by eliminating the subjectivity inherent in reading results of titer methods.

In this study, we have evaluated a commercial rate-nephelometric RF assay, examined the correlation between results by this method (kilo-int. units/L) with tube-titer values, and confirmed the specificity of the assay for Fc-reactive pentameric IgM.

Materials and Methods

Apparatus

The rate nephelometer (Immunochemistry System "ICS") and microsample centrifuge (Microfuge B) were both from Beckman Instruments, Inc., Fullerton, CA 92634 (10).

The RF buffer solution, IgG antigen, and calibrator were from Beckman Instruments, Inc., Brea, CA 92621. The pH 7.0 borate/sodium chloride buffer contained sodium azide (0.2 g/L). The antigen solution contained heat-aggregated human IgG with sodium azide (1.0 g/L). The calibrator solution contained human RF at a concentration near the mid-point of the "A" measuring range (60–400 kilo-int. units/L, see Procedures) plus sodium azide (1.0 g/L). The concentration of RF calibrator was assigned by comparison with the National Reference Preparation for Rheumatoid Factors (lot no. 79-008) from the Centers for Disease Control, Atlanta, GA 30333. Dithiothreitol (DTT), "Sigma" grade, was obtained from Sigma Chemical Co., St. Louis, MO 63178. Rheumaton RF slide kits were obtained from Wampole Laboratories, Cranbury, NJ 08512. We also used the following tube-titer kits: Rapi/TEX RF (Calbiochem-Behring, La Jolla, CA, 92037), Rheumatoid Factor (ICL Scientific, Fountain Valley, CA 92708), and RA Test (Hyland Diagnostics, Deerfield, IL 60015). Kits for IgG and IgM assay were obtained from Beckman Instruments, Inc., for analysis with the Beckman ICS. Vacutainer blood-collection tubes were from Becton Dickinson and Co., Rutherford, NJ 07070.

Procedures

Sample collection. Blood samples were collected in standard red-top Vacutainer Tubes, allowed to clot, and the serum was removed for immediate assay or for freezing (−20 °C) if the analysis was to be done later.

ICS RF measurement. Heat-labile agglutinators of the IgG reagent, presumably the complement C1q, were inactivated by placing 0.5 mL of each patient's serum and calibrator in a
water bath at 56 ± 1.0 °C for 30 min. The mixture then was clarified by centrifugation at 8000 X g. The supernate, undiluted or diluted six- or 36-fold in RF buffer, are respectively referred to as the "A," "B," and "C" dilutions, and cover the following ranges (kilo-int. units/L): "A," 60-400; "B," 360-2400; and "C," 2160-9999, as determined with the ICS RF assay. In routine testing, we prepared the "B" and "C" dilutions only if, on testing, the undiluted serum showed RF activity exceeding the "A" measuring range.

All electronic and rate-measurement factors are programmed into the ICS by inserting into the instrument one of the binary coded cards attached to every bottle of RF antigen and RF calibrator. The RF antigen bottle also contains a fluorescent dye, which acts as a trigger to activate the rate-monitoring functions. The RF antigen card enters curve-fitting parameters that define the relationship of rate units to kilo-int. units of rheumatoid factor per liter for that lot number of reagents. The calibrator, run in duplicate before the unknowns are run, yields a "raw" rate value, which is compared by the system to a target rate, yielding a calibration factor. The reaction rates measured for unknowns are modified by this factor to produce RF concentrations in kilo-int. units/L.

To perform the assay, aspirate a 100-μL aliquot of patient's serum that has been appropriately diluted with a 1:6 fixed-ratio diluter, and add it to the special reaction cell, plus 500 μL of RF buffer. Place the reaction cell, which contains a magnetic stirring bar, into the cell compartment of the ICS. The measurement starts when 42 μL of RF antigen is added to the reaction cell and ends when the instrument verifies a peak reaction rate and displays the RF concentration of the patient's serum. All heat-inactivated samples were either run within 3 h or discarded.

Quantitative latex-agglutination tube-titer methods. The ICL Scientific, Calbiochem-Behring, and Hyland Diagnostics tube-titer methods all involve use of human IgG-coated latex particles as antigen. We followed the exact protocols of the vendors in all cases.

Qualitative erythrocyte-agglutination slide method. Using 20-fold diluted serum, we performed the Wampole rabbit IgG-coated erythrocyte agglutination slide method as described by the vendor.

Results

Precision. Within-run precision was evaluated by testing 20 times each three pooled samples having RF concentrations between 60 and 400 kilo-int. units/L. The mean values of 145 (SD 5.1), 222 (SD 3.3), and 347 (SD 5.7) had coefficients of variation of 3.5, 1.5, and 1.6%, respectively. The between-run precision, as determined by assaying one sample in 20 different runs during one day, was 270 (SD 6.9) kilo-int. units/L (CV 3.1%).

Stability. Because it may not always be feasible to perform the analysis on the day of collection, we investigated the stability of RF as determined by the ICS method in samples that had been stored frozen. Serum specimens from two patients were aliquoted into seven snap-cap plastic tubes and stored frozen at −20 °C. On seven consecutive days one tube of each patient's sample was allowed to thaw at room temperature, mixed well, and assayed for RF. The respective results were 89 (SD 3.4) and 346 (SD 6.6) kilo-int. units/L (CVs of 3.9% and 1.9%).

Comparative analytical sensitivity. We investigated the sensitivity of the Beckman RF assay, which involves use of a human IgG antigen, by comparing results with those by the widely used Wampole slide method, in which rabbit IgG antigen is used. Of 101 samples that were ICS RF-negative, seven were positive by the Wampole slide method. Conversely, of 110 ICS RF-positive samples, 12 were negative by the Wampole slide method. RF results <60 kilo-int. units/L are judged to be negative or "Out of Range Low" on the ICS.

Clinical sensitivity and specificity. Patients who were classified as having mild rheumatoid disease by history, examination, and at least one positive RA-slide test from the referring hospital or laboratory were evaluated by the ICS-RF and Wampole slide methods. Of these 85 clinically positive patients, 82 and 80, respectively, were positive by the vendors' criteria. We also assayed by both methods samples from 85 patients judged clinically negative for rheumatoid disease; 84 and 81, respectively, were negative by the ICS and Wampole methods. These results show that the ICS and Wampole assays have respective sensitivities of 96.5 and 94.1%, and specificities of 98.8 and 95.3% for this population of patients (11).

Linearity. To evaluate the linearity of the ICS method, we diluted twofold with RF buffer a patient's sample whose RF concentration was above the normal measuring range (60 to 400 kilo-int. units/L), to yield a concentration at the upper limit of the "A" dilution measuring range. Then 100, 90, 80, 70, 60, 50, 40, 30, and 20% dilutions of that twofold dilution were made with RF buffer and assayed in duplicate. The results of these analyses are shown in Table 1. We diluted 190 patients' specimens with ICS-RF values exceeding 200 kilo-int. units/L as above and reassayed. Of these samples 71% showed some nonlinearity in RF values measured. The magnitude of the difference between measured and expected RF values for the nonlinear specimens ranged from a low of −14.2% to a high of +92.6%. The most severe nonlinearity usually occurred after the first or second dilution. Repeat analysis of undiluted samples (selected without conscious bias) that yielded linear and nonlinear results confirmed the original analyses in all cases. In addition, the linearity of the RF assay from the highest dilution range "C" to lowest "A" was evaluated by measurement of ICS-RF concentrations in five serial twofold dilutions of 15 patients' samples having RF values ranging from 4230 to 5920 kilo-int. units/L in the "C" dilution range (also selected without conscious bias). Of these sera, 87% showed some nonlinearity. The difference between measured and theoretical in the last twofold dilution ranged from 4.3 to 280%.

Correlation. To evaluate the correlation of the ICS IgM-RF results with titer, we assayed 100 ICS RF-positive samples by the Calbiochem-Behring tube dilution method. Because of the ±1 tube precision limitation on tube dilution assays for RF, any point could actually fall within a large domain. This prevents the calculation of an accurate regression line for comparison with the more nearly precise results from the ICS.

<table>
<thead>
<tr>
<th>Table 1. Linearity</th>
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<tr>
<td>Dilution, %</td>
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</tr>
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<td>100</td>
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<tr>
<td>90</td>
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<tr>
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The percent deviation of ICS-measured RF values from expected values for a patient's sample whose RF concentration was initially above the "A" (60 to 400 kilo-int. units/L) measuring range. The specimen was diluted twofold to bring it into the "A" measuring range, and RF concentrations were measured for the percent dilutions described above.
Fig. 1. Plot of 100 patients' results, RF-positive by the Beckman ICS (kilo-int. units/L), vs Calbiochem-Behring tube-titer values. The ICS designates all measured values for RF less than 80 kilo-int. units/L as "Out of Range Low" (ORLO). The comparatively high imprecision of the titer method causes uncertainty about any data point's exact location in the y-axis, thus precluding the calculation of a precise regression line through the data. The shaded area approximates the ±1 tube range of the titer method, to illustrate this point.

The correlation of these two methods (Figure 1) illustrates through the use of a broad, shaded band that any of the points may vary considerably in their location at any one time.

Patients under clinical treatment or observation for rheumatoid disease often have the concentration of serum RF measured on each office visit. Large analytical variation in these analyses could affect patient management. Therefore, we also investigated the effect of tube titration imprecision on RF results. Blood from one patient with a high RF result and one patient with a low RF result was collected and tested at approximately one-week intervals for seven weeks, during which no clinical changes in rheumatoid activity were noted. Each serum specimen was assayed by the ICS RF method, and by the Calbiochem-Behring, ICL Scientific, and Hyland Diagnostic latex-agglutination tube-dilution methods. The results are shown on Figure 2.

Specificity for pentameric IgM-RF. The specificity of the ICS RF assay for pentameric IgM-RF was assessed by treating aliquots of two positive samples with equal volumes of isotonic saline or DTT (10 mmol/L) and re-assaying for RF concentration. The DTT reagent reduces the pentameric IgM to the monomeric form, which theoretically prevents the formation of light-scattering complexes (12). The continued monomeric Fc immunoreactivity in both sets of samples was verified by the Beckman ICS IgG and IgM assays (Table 2).

Synovial fluid RF analysis. Synovial fluid samples from 10 patients were assayed by the Calbiochem-Behring, ICL Scientific, and Hyland Diagnostics tube-dilution methods and the ICS RF procedure. The results are shown on Figure 3.
and ICS RF methods suggests that the two methods have equivalent sensitivities. The ICS method showed greater sensitivity and specificity than the Wampole slide assay in a clinically defined population of patients. We caution, however, that the actual predictive value of any RF assay, being highly dependent on the incidence of rheumatoid disease in the particular patient population under study, should be determined by each laboratory performing RF analyses (11).

The ICS RF assay was found to have excellent linearity, as seen in Table 1. However, many patients’ samples demonstrated marked nonlinearity in at least one dilution point, both within the “A” (60 to 400 kilo-int. units/L) dilution range (71%) and when serially diluted from the “C” (2160 to 9999 kilo-int. units/L) dilution range (87%). This phenomenon can, we believe, be attributed to the comparatively atypical binding of RF with patient IgG antigen, as suggested by several others (13, 14). Our experience indicates that this occurs with varying severity in each patients’ serum. Thus, some serum specimens may show excellent linearity from 8000 to 200 kilo-int. units/L, whereas others in the same RF concentration range show good linearity only after several serial dilutions in RF buffer. However, even the specimens exhibiting the greatest nonlinearity yielded results that, when multiplied by the appropriate dilution factor, were within a rather small portion of the range represented by a tube-dilution assay at any given RF concentration. The reproducible heat inactivation of C1q complement, which has been shown to interfere with RF assays, is critical to the analytical reliability of the ICS RF assay (4). Because this complement component may reactivate within 3 to 4 h, the analyses for RF should be completed within 3 h, or the samples discarded. Reheating a sample leads to unpredictable and generally erroneous RF results.

Assay of 100 patients’ samples by both the Calbiochem-Behring tube-dilution method and the ICS RF assay showed general agreement (Figure 1). The within-run precision of the ICS RF value on the x-axis contrasts with the broad ±1 tube uncertainty of any tube-titer point on the y-axis. The broad shaded band through the data in Figure 1 calls attention to our belief that, although Figure 1 shows a trend of increasing tube titer with increasing RF in kilo-int. unit/L, no precise analytical correlation is possible, owing to the great inherent imprecision of the tube-dilution methods as shown in recent national quality-control surveys (5, 7). Because RF methods may act on a given patient’s serum with different specificities and sensitivities, the traditional correlation studies involving many different patients’ samples superimpose variables that tend to make it difficult to estimate results for a patient accurately. If instead, individual patients are followed over several weeks with different analytical RF methods, the problem of variation of an assay’s performance parameters is reduced, as shown in Figure 2 for patients CB and VH. In both cases the patients involved reported unchanged symptoms during the seven weeks of observation. The ICS-RF values remained comparatively stable while the tube-titer results fluctuated. Patient CB exhibited an increase in RF of approximately 25% between weeks four and five. Although analytically significant and reproducible, the clinical significance of the change in RF is unclear. The patient did not report changes in symptomatology during this seven-week period, but the increase may have foretold an increase in disease intensity later than our study period at week twelve. Also as shown in Figure 2 these titer fluctuations corresponded to marked changes in corresponding RF concentration expressed as kilo-int. units/L, and often moved in different directions. This further suggests that it may not be appropriate to compare a precise new method with established methods of relative imprecision.

The specificity of the ICS RF assay for pentameric IgM was

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**Table 2. ICS RF Test Specificity for Pentameric IgM**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IgM-RF</th>
<th>IgG</th>
<th>IgM</th>
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<tbody>
<tr>
<td>Patient A</td>
<td>4050</td>
<td>910</td>
<td>910</td>
</tr>
<tr>
<td>CBDT</td>
<td>1452</td>
<td>7300</td>
<td>940</td>
</tr>
<tr>
<td>Patient B</td>
<td>500</td>
<td>500</td>
<td>550</td>
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* Split samples from two patients were treated with either dithiothreitol (DTT), 10 mmol/L, which disrupts the pentameric character of IgM, or with isotonic saline, and assayed on the ICS for IgM-RF (kilo-int. units/L), IgG (mg/L), and IgM (mg/L). The ICS indicates “Out of Range Low” (ORLO) when RF is less than 60 kilo-int. units/L.

**Monitoring the efficacy of clinical treatment.** The usefulness of the ICS-RF assay in monitoring the concentrations of serum RF in a patient as a possible measure of efficacy of treatment was investigated. Patient FC, a 56-year-old woman who presented with a history of recurrent rheumatoid disease symptoms but otherwise generally good health had an initial ICS-RF concentration of 8940 kilo-int. units/L. Conservative management with rest, exercise, heat, and aspirin (5 g daily) was begun, and blood samples for RF measurement were drawn at two-week intervals. The serum RF values fell to 5620 kilo-int. units/L by the third visit but remained between 5000 and 7000 kilo-int. units/L for the next three months (six visits). Failure of the patient’s symptoms substantially to decrease and the apparent plateauing of serum RF concentration suggested the need for additional therapeutic action. Gold salt (gold thiomalate) therapy was initiated. The serum RF decreased to 2820 kilo-int. unit/L after the first four weekly intramuscular injections. After four months of therapy with gold the RF concentration had dropped to the 200 to 400 kilo-int. unit/L range, with nearly complete abatement of pain and discomfort in the patient.

**Discussion**

The Beckman ICS RF assay showed excellent within-run and between-run precision, with CVs of less than 4.0%, even for low concentrations of RF. There was no measurable loss of RF in samples frozen for as long as seven days after collection. The comparable distribution of apparent false positives and negatives in patients’ samples for both the Wampole slide...
confirmed by the lack of RF activity in dithiothreitol-treated samples as shown in Table 2. Synovial fluid is typically more viscous and turbid than normal serum, but is not infrequently submitted for RF quantification. As shown in Figure 3, the ICS RF assay compared favorably with the tube-dilution methods, in that they all indicated that the samples were RF positive.

The clinical utility of the ICS-RF assay for patient follow-up over prolonged periods was very encouraging. The case of patient FC was followed for seven months. ICS-RF analyses of serum samples drawn every two weeks during this period correctly indicated the relative efficacy of treatment through correlation with the severity of symptoms reported by the patient. Because of the high analytical variability of tube titer RF values, much importance has not been given to the format used for reporting assay results. We have found that plotting the patient's ICS-RF results on a progressive summary report (semilog scale) provides a good presentation of the more analytically precise RF values, with cognizance of the analytical variations that can occur between different samples. The semilog scale tends to reduce the effect of the previously mentioned biological interference of IgG in some specimens without diminishing the clinical utility of the results. The presentation of the ICS-RF concentrations for patients CB and VH in Figure 2 is an example of this reporting method.

We found the Beckman rate-nephelometric RF assay easy to perform, precise, and capable of linearity over a wide RF concentration range. The assay correlated with tube titer in the same general fashion as described earlier (8). However, the increased reliability of this assay should permit a clearer monitoring of RF concentration with changes in treatment and disease course than is now possible with tube-dilution methods.

References