Determination of Human Immunoglobulin M Rheumatoid Factor by a Solid-Phase Radioimmunoassay Which Uses Human Immunoglobulin G in Antigen-Antibody Complexes

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A solid-phase radioimmunoassay for the rapid determination of human immunoglobulin M (IgM) rheumatoid factor (RF) has been developed. Preparation of the solid phase for the assay involved the formation of complexes between respiratory syncytial virus-specific human IgG antibodies and virus antigen on the surface of polystyrene balls. Binding of serum RF to IgG in the immune complex was subsequently detected by 125I-labeled mu-chain-specific antibodies to human IgM. The amount of radioactive indicator antibody bound was converted to units of RF by comparison to the standard curve for an RF reference serum pool. This assay should prove useful in studies of the physiological role of RF, since it can effectively measure low levels of circulating RF.

Diagnosis of a recent virus infection can be accomplished by demonstration of virus-specific immunoglobulin M (IgM). For this purpose, we have recently developed solid-phase radioimmunoassay (RIA) methodology for the detection of the IgM antibody response in rubella, herpes simplex, and measles virus infections (2, 12, 13, 19). When testing for IgM antibodies, however, the possibility of false positive results caused by the presence of IgM-class rheumatoid factor (RF) must be considered. It has been shown, for example, that RF can give misleading results in the indirect immunofluorescence test for IgM antibodies (23). An understanding of how the presence of RF might affect the viral IgM RIAs was therefore necessary before these assays could be routinely applied. Undertaking such an evaluation, however, required a method for RF determination that would be simple, yet comparable in sensitivity to that of the viral IgM antibody RIAs.

RF interference in an IgM antibody test arises through secondary binding of RF to complexes of the test antigen and specific IgG. Consequently, RF binding to human IgG in an antibody-antigen complex was used as the basis of the RF RIA described here. The idea of using human IgM immunologically complexed with antigen to demonstrate RF is not new, since Rh D+, group O erythrocytes sensitized with incomplete anti-D sera have been used as an indicator system for RF (26). This assay was limited in application, however, due to lack of anti-D sera that were useful for sensitizing erythrocytes (8). A lack of sensitizing sera is not a concern with the present RF assay, because it was found that sera from randomly selected adult blood donors could be used for this purpose.

MATERIALS AND METHODS

Specimens. Serum samples were obtained from 61 patients (13 males, 48 females) having a clinical diagnosis of definite or classical rheumatoid arthritis (RA) according to the American Rheumatism Association criteria (22). An RF reference serum pool was made from sera of 12 of these patients. Control sera were collected from 46 virology department staff members (13 males, 33 females), 110 male blood donors, and 111 female blood donors. The blood donor specimens were obtained through the courtesy of the Finnish Red Cross Volunteer Blood Donor Center, Turku. All RA patients and controls were 18 years of age or older. The sera were kept at −20°C until tested.

RS virus antigen. Antigen was prepared from Vero cells infected with the Randal strain of respiratory syncytial virus (RS) virus. The Vero cells were maintained in Eagle minimum essential medium supplemented with 5% tryptose phosphate broth and 0.2% bovine serum albumin, fraction V, and containing 200 IU of penicillin and 200 μg of streptomycin per ml. Extensive syncytia formation was observed approximately 5 days after infection with a low multiplicity of virus. The medium was replaced with 0.02 M sodium phosphate (pH 7.4) containing 0.14 M NaCl (PBS). and the infected cells were dislodged by scraping. They were then collected by low-speed centrifugation, resuspended in PBS, and homogenized at 4°C at maxi-
ium speed for 1 min in a Sorvall Omnimixer. After the homogenate was centrifuged at 80,000 × g at 4°C for 60 min, the pelleted material was resuspended in PBS by sonic disruption at 4°C. The protein concentration of the antigen solution was determined by the Lowry method (18), using bovine serum albumin as a standard. The antigen was stored at −20°C; upon thawing, it was again sonically disrupted to obtain a homogeneously diluted suspension.

Routine tests. The latex particle agglutination test for RF (Behringwerke, Marburg, W. Germany) was adapted to V-bottomed microtiter plates. An equal volume of 1:5-diluted latex particles was added to dilutions of serum specimens, giving a further twofold dilution, and the test was read after 2 h at room temperature.

RF detection by agglutination of rabbit IgG-sensitized sheep erythrocytes (the Waaler-Rose test) was performed by a microtechnique after preabsorption of the sera with normal sheep erythrocytes (1, 24).

The RS virus complement fixation test was performed by a standardized microtechnique (5).

Isolation of IgG. IgG was isolated from pooled sera of five patients convalescent from RS virus infection and pooled sera of eight randomly chosen staff members. The RS virus patients’ sera were selected from specimens tested in our virus diagnostic laboratory. Following precipitation of total immunoglobulins with 18% Na2SO4, the IgG fractions were obtained by chromatography on diethyliaminomethyl-Sepharose (Pharmacia, Upsala, Sweden) equilibrated with 0.01 M sodium phosphate (pH 8.0). Protein not retained on the resin was taken to be IgG, and concentrations were determined using 13.8 as the absorbance at 280 nm of a 1% solution in a 1-cm cuvette. Storage was at −20°C.

Antisera. Porcine antisera specific for the gamma chain of human IgG or the mu chain of human IgM were obtained from Orion Diagnostica (Helsinki, Finland). The gamma-chain-specific antibodies were isolated from immunosorbent chromatography on a column of AH-Sepharose (Pharmacia) to which human IgG had been coupled (3). The mu-chain-specific antibodies were similarly isolated on an IgM immunosorbent column, except that any residual anti-IgG antibodies were first removed by passing the antiserum through an IgG immunosorbent column. Specifically bound antibodies were eluted by 3 M NaSCN, then immediately dialyzed at 4°C against PBS, and finally stored at −60°C.

Iodination with Na125I (Amersham, England) was done by a chloramine T method (10), except that NaHSO3 was omitted (16). The reaction was stopped, and separation of the iodinated antibodies from unincorporated iodine was achieved by column chromatography as described previously (28). Specific activities of 5 to 20 μCi/μg were routinely obtained. The iodinated antibodies remained immunologically active for at least 8 weeks when stored at 4°C in PBS containing 20% glycerol, 1% bovine serum albumin, and 0.1% Na2NO3.

Preparation of the solid-phase human IgG-RS virus antigen complexes. Specular-surface polystyrene balls, 6.4 mm in diameter (Precision Plastic Ball Co., Chicago, Ill.), were used as the solid phase for the assay. The balls were first incubated at room temperature in PBS containing 7 to 8 μg of RS virus antigen per ball. After 16 to 18 h, the buffer containing unadsorbed antigen was removed, and the balls were air dried at room temperature to fix the antigen firmly to their surface. Fixation by organic solvents was found to be detrimental. The antigen-coated balls were then incubated at room temperature for 20 to 24 h in PBS containing 0.5% bovine serum albumin, 0.5% Tween 20, 0.1% Na2NO3, and 4 μg of IgG, isolated from the serum pool from RS virus convalescent patients, per ball. Balls to be used immediately were removed and air dried at room temperature, and those that were prepared in excess were stored at 4°C in the IgG solution and dried as required. Although drying of the balls at this stage was employed to facilitate handling during the assay of large numbers of specimens, no differences were seen when the balls were used wet. Balls prepared in this manner have been stored up to 2 months and remained fully functional.

RF assay procedure. PBS containing 20% pig serum, 2% Tween 20, and 0.2% Na2NO3 was used as the assay diluent. It was stored at −20°C; after thawing it was heated at 65°C for 30 min, filtered through Whatman no. 1 paper, and used for 2 days only. Each serum specimen was diluted 1:200 and tested in duplicate. Samples of 200 μl were placed in disposable plastic tubes (12 by 70 mm), and a polystyrene ball with preformed immune complexes on the surface was added to each tube. The RF reference serum pool and appropriate buffer blanks were included in each assay. Following incubation at 37°C for 1 h, unbound proteins were aspirated and the balls were washed twice with 5 ml of tap water. RF bound to the IgG in the immune complexes on each ball was detected by incubation at 37°C for 1 h in 200 μl of 125I-labeled mu-chain-specific antibodies to human IgM. Unbound indicator antibody was removed, and, after washing twice with 5 ml of tap water, each ball was rolled into a clean tube for counting in an LKB Wallac 1280 gamma-ray counter. After correcting for the background counts per minute of the buffer blanks, the counts per minute for each serum was expressed as a percentage of the counts per minute of the RF reference serum pool. Conversion to units of RF was by comparison with the standard curve obtained by assaying dilutions of the RF reference serum pool to which an arbitrary value of 1,000 RF units had been assigned. If a serum specimen was found to have more than 500 units when tested initially, it was diluted 1:10 and retested. Where presented graphically, RF unit values have been rounded to the nearest integral divisible by 5.

The amount of 125I-labeled mu-chain-specific antibodies to human IgM used per assay tube was standardized twice a week to compensate for variation in iodinations and for radiolysis inactivation of the indicator antibodies. IgM was isolated from human serum by sodium sulfate precipitation followed by chromatography on G-200 Sephadex (Pharmacia) equilibrated with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) containing 0.15 M NaCl and 0.1% Na2NO3. The IgM was adsorbed onto polystyrene balls by incubation of balls overnight at room temperature in PBS containing 5 μg of IgM per ball. Buffer and unadsorbed IgM were removed, and the balls were
air dried and stored at 4°C. The IgM-coated balls were incubated at 37°C for 1 h in 200 µl of iodinated mu-chain-specific antibodies to human IgM in assay diluent and prepared for counting as described above. The absolute amount of radiolabeled indicator antibody required to give 2,500 cpm bound under these conditions was calculated and used per assay tube in the RF RIA.

Removal of RF. A mixture of 5 µl of the test serum and 195 µl of 1:10-diluted IgG-coated latex particles (Behringwerke) was incubated at room temperature overnight. The latex particles were then removed by centrifugation for 5 min in a Beckman Microfuge B, and the supernatant fluid was retained for RF analysis. Sera that contained rubella virus-specific IgM antibodies were selected as controls for the absorption procedure. Detection of rubella IgM antibodies was done as described previously (12, 19), except that PBS containing 0.5% bovine serum albumin, 0.5% Tween 20, and 0.1% NaCl was used as the serum diluent, and Eagle minimum essential medium containing 10% heat-inactivated calf serum, 0.5% lactalbumin hydrolysate, 1% Tween 20, and 0.1% NaCl was used to dilute the iodinated mu-chain-specific antibodies to human IgM.

Statistics. RF levels of RA patients and controls were compared using Kendall's S test. Comparison of the RF RIA with the latex and Waaler-Rose RF tests was by calculation of Spearman correlation coefficients.

RESULTS

Preliminary studies. Polystyrene balls are used as the solid phase of the rubella, herpes simplex, and measles virus-specific antibody RIAs developed in our laboratories (2, 12, 13, 19). Since the viral antigen-coated balls are easily prepared, manipulated, and washed during the RIA procedure, these same balls were chosen as the solid phase for RF RIA.

Initial attempts to develop an RF RIA employed purified human IgG directly adsorbed to the polystyrene balls. Consistently high background binding of the 125I-labeled mu-chain-specific antibodies to human IgM was observed despite attempts to highly purify the human IgG by repeated diethylaminoethyl-Sepharose chromatography after fractionation on G-200 Sephadex, and in spite of the fact that the iodinated mu-chain-specific antibodies did not react with human IgG in complexes with antigen (as evidenced by the low buffer blank values of Fig. 2). Since RF readily binds to IgG in immobilized immune complexes, as demonstrated by its interference in IgM antibody tests (23), the current assay used instead immune-complexed IgG on the solid phase as the RF binding target.

Preparation of the solid-phase immune complexes. A membrane-type, lysate preparation of RS virus-infected cells was chosen as the antigen for the assay because virtually all of the RS virus antigen remains cell associated during replication of the virus in tissue culture (17, 27). This allows easy preparation of large quantities of antigen. In addition, such a membrane-associated antigen has been found to be ideal for the coating of the polystyrene ball solid phase (unpublished results).

The optimal RS virus antigen concentration for coating the polystyrene balls was determined by adsorbing balls with increasing amounts of antigen, followed by drying and incubation with 10 µg of IgG, isolated from the pooled sera of RS virus convalescent patients, per ball. Bound IgG was then detected with 125I-labeled gamma-chain-specific antibodies to human IgG. Coating with more than 10 µg of RS virus antigen per ball did not significantly increase the IgG binding, whereas coating with less than 5 µg per ball gave significantly decreased IgG binding; consequently, 7 to 8 µg of antigen per ball was chosen for coating of the solid phase.

Although IgG isolated from pooled sera of RS virus convalescent patients was routinely used to prepare the solid-phase antibody-antigen complexes, IgG isolated from pooled sera of staff members could have been used as well. Figure 1 illustrates the dependence of RF binding on the concentration of IgG utilized to sensitize the viral antigen-coated polystyrene balls. Equal RF binding was obtained with 4 µg (per ball) of IgG from pooled sera of RS virus convalescent patients and 120 µg (per ball) of IgG from pooled sera of staff members. The 30-fold difference in the IgG concentration required to provide a polystyrene ball with an adequate amount of antigen-complexed IgG is directly related to the RS virus antibody levels of the original serum pools, since RS virus complement-fixation testing showed titers of 1:512 and 1:16 to the RS virus patient and the staff member serum pools, respectively.

125I-labeled gamma-chain-specific antibodies to human IgG were used to determine how uniform the IgG binding was following incubation of the viral antigen-coated balls with 4 µg of IgG from the pooled RS virus convalescent sera per ball. Intra-lot variation was from 3 to 6%, and inter-lot variation was from 12 to 15%. This variation did not affect the assay results, provided the same lot of prepared balls was used for a given assay, since RF levels in unknown sera were expressed relative to the RF reference serum pool that was included in each assay.

Routine assay conditions. Incorporation of 20% pig serum into the assay diluent was to ensure that nonspecific adsorption of IgM in serum specimens to the solid phase did not occur. Pig serum was chosen because the mu-
Chain-specific antisera to human IgM had been produced in swine. The addition of 2% Tween 20 to the assay diluent made weekly repurification of the 125I-labeled mu-chain-specific antibodies unnecessary. With Tween 20 present, iodinated radiolysis breakdown products did not adsorb to the solid phase, and assay buffer blank values remained low. Heat inactivation of the assay diluent at 56°C for 30 min was essential.

Incubation of serum specimens for times greater than 1 h at 37°C did not appreciably increase RF binding. Increased specific binding of the 125I-labeled mu-chain-specific antibodies to human IgM occurred by lengthening the incubation time at 37°C from 1 to 4 h or overnight. However, since 60 to 70% of the maximum indicator antibody binding occurred within the first hour, this incubation time was selected. The 1-h-plus-1-h incubation times readily allow two assays to be run within 1 day.

Dilution curves of RA patient serum specimens having low to high RF levels are shown in Fig. 2. Because patient A.S. had a higher RF level than the RF reference serum pool, a dilution curve was also made for an initial 1:10 dilution of this serum. A serum dilution of 1:200 was chosen for routine assay, since lower dilutions resulted in plateauing of RF binding when sera with high RF levels were tested, and higher dilutions resulted in undetectable RF binding when sera with low RF levels were tested.

Because the solid phase for the assay contains IgG in immune complex form, it was anticipated that complement in the serum specimens would compete with RF for the IgG bound to RS virus. The effect of treating the serum specimens at 56°C for 30 min was therefore tested. This treatment, however, consistently decreased the observed RF levels by 10 to 25% and was thus found to be unnecessary.

Assay standard curve and reproducibility. The RF RIA standard curve (Fig. 3) was obtained by assaying dilutions of the RF reference serum pool and was found to be nonlinear. The disproportionate binding of the 125I-labeled

Fig. 1. Effect of variation in concentration of the IgG used to prepare the solid-phase antibody-antigen complexes. Following incubation of RS virus antigen-coated polystyrene balls with increasing amounts of IgG, the balls were dried and incubated with a 1:200 dilution of the RF reference serum pool. Bound RF was detected with 125I-labeled mu-chain-specific antibodies to human IgM. The IgG was isolated from pooled sera of RS virus convalescent patients (A) and staff members (B). The 4-μg/ball concentration of IgG from pooled sera of RS virus convalescent patients (arrow) was chosen for preparation of the solid-phase immune complexes in subsequent experiments.
mu-chain-specific anti-human IgM antibodies to smaller amounts of RF suggests that the assay is well suited for the detection of low levels of circulating RF. Evidence for this is shown in Table 1. RA patients C.S., V.V., and M.S. were RF negative by both the latex and the Waaler-Rose RF tests. In contrast, with the RF RIA, it was found that patients V.V. and C.S. had 6- and 20-fold higher RF levels, respectively, than did patient M.S.

Table 1 also shows the reproducibility of the RF RIA. Sera from the five RA patients presented in Fig. 2 were assayed on 8 different days. The mean coefficient of variance for the RF unit values of these patients was 0.19, demonstrating that the assay can be used for quantitative comparisons of serum RF levels.

RF levels in patients and controls. The RF levels of RA patients and members of three control groups are presented in Fig. 4. The arithmetic mean of the RF unit titers ± standard error of the mean was 860 ± 202 for RA patients, 10.9 ± 1.9 for staff members, 21.3 ± 8.1 for male blood donors, and 10.7 ± 1.7 for female blood donors. The difference between the RF levels of RA patients and controls was highly significant (P < 0.001). The RF levels of the three control groups did not differ significantly (P > 0.9).

It should be noted here that RF levels found in 24 early convalescent serum specimens from 12 RS virus patients ranged from 5 to 30 units with a mean value of 11.9 units. This demonstrates that the 4 μg of pooled RS virus patient IgG used per antigen-coated ball sufficiently saturated the RS virus antigenic sites so that RS virus-specific IgM could not bind. Consequently, the presence of RS virus-specific IgM antibodies in a serum specimen will not cause a false positive RF result with the present assay.

That the IgM molecules which bound to the IgG in the solid-phase immune complex were specific anti-IgG antibodies was demonstrated by pretreatment of the test sera with human IgG-coated latex particles. Representative data are presented in Table 2. RA patient and control sera originally having ≥20 units of RF were reduced to RF levels of <15 units by this treat-
ment. All sera having <20 units of RF were likewise found to have decreased RF levels after the absorption procedure, although the decreases seen were variable. The virus-specific IgM antibody titers of rubella convalescent sera were unaffected by the incubation with IgG-coated latex particles.

Figures 5 and 6 show the comparison of the RF RIA with the latex and Waaler-Rose RF tests. There was a significant correlation between the RF RIA titers and the latex test titers ($r = 0.75$) and between the RF RIA titers and the Waaler-Rose test titers ($r = 0.76$). A serum was considered RF positive by the latex and Waaler-Rose tests when a positive agglutination reaction was obtained at a dilution of 1:32. However, RF RIA analysis of those sera considered negative by the conventional RF tests, principally the Waaler-Rose test, revealed many to have elevated levels of RF (for example, ≥20 units).

**DISCUSSION**

The laboratory diagnosis of RA, at present, relies mainly on the detection of RF by the latex and Waaler-Rose agglutination tests. Both methods suffer from problems of standardization and reproducibility, and neither test can be effectively used for quantitation of RF, particularly when it is present at low levels. In attempts to achieve better RF measurement, several investigators have reported RF detection by RIA procedures. The first such assay was based on the double-antibody principle (7) and, although sensitive, takes 5 days to complete and requires a filtration step to separate bound and unbound radiolabeled marker IgG; this severely limits the number of samples that can be handled at one time. In a second assay described by the same laboratory (6), IgG coupled to cellulose was used to separate bound and unbound radiolabeled RF, with levels of RF in test samples being measured by the degree of competition observed. Use of cellulose as the solid phase, however, requires several cycles of centrifugation followed by washing to achieve suitable background values.

A more easily handled and washed plastic solid phase (i.e., tubes) has been employed in an RF RIA (9, 20). This assay used rabbit IgG as the RF binding target, however, which would not seem to be ideal since only a variable amount of human RF reacts with rabbit IgG (21).

Two additional reports on RIA of RF have more recently appeared. Both human Fc fragments and intact human IgG adsorbed to plastic tubes were used as the RF binding target in the first (4), and human IgG fixed in the wells of microtiter trays was used for the same purpose in the second (14). One difficulty with both assays appears to be the problem encountered during our attempts to use IgG directly adsorbed to polystyrene balls as an RF binding target, i.e., high background binding of the $^{125}$I-labeled mu-

### Table 2. Removal of RF with human IgG-coated latex particles

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<th>After absorption</th>
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* The rubella virus-specific IgM titer of this specimen was 1:8,000 both before and after the absorption procedure.

**Fig. 5. Comparison of the RF RIA and the latex agglutination test; n = 61.**

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chain-specific indicator antibodies to human IgM. One possibility for this problem is that residual IgM is contaminating the IgG preparations used (14). Based on the present results, however, an alternative explanation can be given. IgG cross-reacting antibodies are most often removed from anti-human IgM antisera by immunabsorbent chromatography in which IgG, covalently coupled to a support such as Sepharose, is used. This removes all antibodies which cross-react with IgG in an aqueous environment and therefore in a relatively native form. When IgG is adsorbed to polystyrene by strong hydrophobic bonds, however, there may be induced conformational changes in the molecule, exposing new antigenic sites which can still cross-react with what is believed to be monospecific anti-human IgM or mu-chain-specific anti-IgM antisera. Support for this view comes from the finding that changes do occur in the conformational and immunogenetic properties of human IgG when adsorbed to polystyrene particles (15).

Non-specific binding of the radiolabeled mu-chain-specific antibodies to human IgM in the present RF RIA was approximately 0.1% of the input radioactivity; assay buffer blanks were always less than 100 cpm and consistently one-half of that. The assay also has other advantages. It can be established with reagents easily prepared in large quantities and, once established, can be used to test on the order of 100 samples at one time if necessary. The use of human IgG as the RF binding target, rather than IgG from another animal species, fulfills at a molecular level what would seem to be a logical requirement for any assay designed to detect human RF. Furthermore, the binding of RF to IgG in an immune complex form may closely reflect the situation in vivo, where RF synthesis is most likely an immunological response to IgG-antigen complexes (11, 25).

Although 125I-labeled mu-chain-specific antibodies to human IgM were used to obtain the reported results, these can readily be replaced by enzyme-conjugated indicator antibodies (unpublished data), allowing application of the test in laboratories where radioactive isotopes are prohibited or where expensive gamma irradiation counters are not available. It should be noted that the principle upon which the assay is based does not allow for detection of IgG-class RF. In any event, detection of IgG-class RF may remain of research interest only, since circulating levels in RA patients negative by the latex test are not sufficiently different from those in controls to make IgG-class RF measurement of diagnostic value (4). Should IgG-class RF detection be of interest, however, the best available method appears to be the use of human Fc fragments on an appropriate solid phase in conjunction with radiolabeled or enzyme-conjugated Fab-specific anti-human immunoglobulin indicator antibodies (4).

The present data support the view that not only RA patients but also the majority of normal controls possess circulating RF. Since these RF levels were detected in the presence of 40-fold excess of pig serum proteins and were reproducible, and since they could be virtually eliminated or at least significantly reduced by human IgG-coated latex particle absorption, it appears that a true RF activity was being measured in each case. Although it will be difficult to obtain more definitive evidence for low levels of circulating RF, the data presented bring into question whether or not there are true RF-seronegative RA patients or, for that matter, individuals. It perhaps is not surprising that all persons should have circulating RF since the exact biological function of RF still remains a mystery, and in ordinary circumstances RF may perform a beneficial function for the individual. Only in certain disease states, RA being one, does RF synthesis then escape normal controlling mechanisms due to as yet unknown stimuli.

Since the assay described herein is well suited for the measurement of low levels of RF or the detection of small changes in RF levels of serially collected serum specimens, it should prove use-
ful in research concerning the possible physiological role of RF. Matters of more clinical interest such as minor changes in RF levels during the disease course, whether RA or otherwise, and the effect of therapy on RF levels will also be amenable to study. Finally, the availability of an RF assay based on similar technology and having a sensitivity comparable to that of our virus-specific IgM antibody RIAs will now enable us to quantitatively assess the effect that the presence of RF may have on these diagnostic tests.

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LITERATURE CITED