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Solid-Phase Radioimmunoassay of Rubella Virus Immunoglobulin G and Immunoglobulin M Antibodies

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A solid-phase radioimmunoassay method has been developed for the detection of rubella virus-specific immunoglobulin G (IgG) and IgM antibodies in human serum specimens. Purified rubella virus was adsorbed onto polystyrene balls, and antibodies that attached to the virus-treated balls were detected by subsequent binding of ^{125}I-labeled anti-human gamma or anti-human mu immunoglobulins. A total of 77 serum specimens were tested. Binding ratios between positive and negative sera were as high as 22 in the IgG assay but rarely exceeded 3 in the IgM assay. The sensitivity of the IgG assay was found to be 16 to 256 times higher than that of the rubella virus hemagglutination inhibition test. The IgG radioimmunoassay can be readily adopted for routine diagnostic use. The IgM radioimmunoassay, however, due to its lower sensitivity, must be modified before being routinely applied.

The accurate diagnosis of rubella virus infections is a major concern of virus diagnostic laboratories. Virus hemagglutination inhibition (HI) and complement fixation (CF) tests are widely used for this purpose. The results obtained, however, are not always conclusive. One problem occasionally encountered is whether a low HI titer is due to a rubella infection or to incomplete removal of nonspecific hemagglutination inhibitors from the serum (15). Another problem arises from the times serum specimens are collected after appearance of clinical rubella symptoms. If the first specimen is collected later than 4 or 5 days after onset of rash, a significant rise in rubella antibody titers between the first and subsequent samples often cannot be detected (21). This makes diagnosis of a recent infection difficult.

In uncomplicated and postnatal rubella infections, specific immunoglobulin M (IgM) antibodies are detectable for only 3 to 8 weeks after onset of rash (20, 21). Thus, if rubella-specific IgM antibodies can be demonstrated, a recent infection is indicated. Several methods have been proposed for detecting such antibodies, including separation of IgM and IgG by sucrose density gradient centrifugation or column chromatography followed by HI testing of the separated immunoglobulins (9, 11, 32), reduction of IgM with 2-mercaptoethanol with subsequent comparison of HI titers of treated and untreated serum (2), *Staphylococcus aureus* absorption of IgG from serum samples followed by HI testing of the IgM-containing supernatant (1), and detection of IgM antibodies by indirect immunofluorescence (5, 12, 20). Most virus diagnostic laboratories have not, however, found any of these techniques consistently reliable or practical enough for the testing of large numbers of serum specimens.

In contrast, detection of viral antigens and viral antibodies by radioimmunoassay (RIA) techniques is highly sensitive and specific (10, 16, 18, 19, 24-27, 29, 30, 35). Furthermore, a refinement of the RIA technique has involved the detection of class-specific viral antibodies (4, 6-8). Recently, a solid-phase RIA for IgM and IgG class antibodies against bovine serum albumin (BSA) in chickens was described by Viljanen et al. (33); BSA was coupled onto a paper disk and antibodies trapped by the antigen were detected by their capacity to bind ^{125}I-labeled anti-chicken mu or anti-chicken gamma immunoglobulin. In the present work, this method was further developed for the assay of virus-specific human antibodies, using polystyrene balls as the solid phase. Presented in this paper is a practical and reliable RIA procedure for detecting rubella virus-specific IgG antibodies in serum specimens. Also included are preliminary results for detection of rubella-specific IgM antibodies using the same methodology.

MATERIALS AND METHODS

Antigen purification. The RA 27/3 strain of rubella virus was grown in suspension cultures of BHK-21/13S cells (13). The cultures were harvested

at 3 days postinfection, and cells were removed by low-speed centrifugation. The supernatant was clarified by centrifugation at 8,000 × *g* for 15 min, and the pH was immediately adjusted to 8.0. An Amicon 402 apparatus containing an XM-300 membrane was then used at 4°C to concentrate the virus solution approximately 15- to 20-fold. Virus was recovered from the Amicon concentrate by pelleting through 15 ml of 20% sucrose in TSE buffer (0.01 M tris-(hydroxymethyl)aminomethane, 0.15 M NaCl, and 0.002 M ethylenediaminetetraacetate, pH 9.0) onto a 3-ml layer of 60% sucrose in TSE buffer. Centrifugation was for 2 h at 25,000 rpm in a Spinco SW27 rotor at 4°C. Sucrose was removed by dialysis against TSE buffer. The hemagglutination titer of the purified virus varied from 64 to 512, and the protein content, determined by the method of Lowry et al. (22), was 300 to 1,000 μg/ml.

Serum specimens. A series of serum specimens was obtained from six army trainees who had developed an acute clinical rubella infection with significant increases in HI and CF antibody titers. A total of 26 specimens was collected. Other sera consisted of 51 specimens selected from our virus diagnostic laboratory. Included were 32 specimens that were negative in the rubella HI test and 19 specimens that had HI titers of 128 or less. All serum specimens were stored at −20°C until used.

^{125}I-labeled anti-human gamma and anti-human mu immunoglobulins. Human IgG and IgM were purified as described by Viljanen et al. (33). Briefly, human serum immunoglobulins were precipitated with sodium sulfate and chromatographed on Sephadex G-200. IgM fractions were twice recycled on Sephadex G-200. IgG fractions were further purified by diethylaminoethyl-cellulose chromatography. In immunoelectrophoresis and immunodiffusion tests against anti-human sera, the IgG and IgM preparations each gave only one precipitation arc. These preparations were then used to immunize sheep. The immune sera obtained were purified by the use of immunoabsorbents (33). The final preparations of anti-human gamma and anti-human mu immunoglobulins were free of other serum proteins and antibodies.

The anti-immunoglobulins were labeled with ^{125}I-isotope according to the method of Hunter and Greenwood (17), with modifications according to Viljanen et al. (33). Specific activities ranged from 30 to 100 μCi/μg. After iodination, the anti-immunoglobulin preparations were supplemented with 1% normal sheep serum. Degradation products resulting from radioactive decay were removed weekly by chromatography on Sephadex G-200. Peak fractions were pooled and stored at 4°C until required. Under these conditions, the labeled anti-immunoglobulins were found to remain active for 4 to 8 weeks.

RIA procedure. Purified rubella virus was adsorbed onto polystyrene balls (6.4 mm in diameter; Precision Plastic Ball Co., Chicago, Ill.) by incubating balls submerged in an antigen solution at room temperature for 16 h. The protein concentration of the rubella antigen solution was 110 μg/ml, unless otherwise indicated. Phosphate-buffered saline (PBS), pH 7.35, was used as diluent. After incubation, the balls were air dried and stored at 4°C.

Fourfold serial dilutions of serum specimens were pipetted in 0.2-ml volumes into 4-ml disposable plastic tubes (Nunc Products, Roskilde, Denmark). The serum samples were diluted with PBS, pH 7.35, containing 1% BSA, fraction V (1% BSA-PBS). A polystyrene ball with adsorbed rubella antigen was then added to each tube. Buffer blanks (balls incubated with 1% BSA-PBS and no serum) and a titration of rubella antibody-positive and -negative control sera were included in each test.

After incubating at 37°C for 1 h, the serum dilutions were aspirated off and the balls were washed twice with 5 ml of tap water. A 0.2-ml volume of ^{125}I-labeled anti-human gamma or mu immunoglobulin was then added to each tube. The labeled antibody preparations were diluted to 30,000 cpm/0.2 ml with Eagle minimum essential medium supplemented with 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and antibiotics as described by Rosenthal et al. (26). After incubation at 37°C for 1 h, the radioactive solutions were aspirated off and the polystyrene balls were washed as described above. The balls were then placed in clean plastic tubes, and bound radioactivity was counted in an LKB Wallac 1280 gamma counter.

CF and HI tests. Rubella antigen was prepared for the CF test according to the alkaline extraction procedure described by Halonen et al. (13). Sever's microtechnique (28) was used, with veronal-buffered saline containing 0.1% gelatin used as diluent. The optimal dilution of antigen was estimated according to the standardized CF technique (3). Standardization of erythrocyte suspensions was done as in the HI test (31). For the HI test, rubella hemagglutinin was prepared in BHK-21/13S cells maintained in a medium containing BSA and no serum (14). HI tests were performed by microtechnique according to the modified test used at the Center for Disease Control, Atlanta, Ga. (31). CF and HI titers are expressed as reciprocals, except in Tables 1 and 2, where $\log_2$ values are used.

RESULTS

RIA solid phase. Although data presented in this report were obtained from experiments in which polystyrene balls were used, first attempts to establish a rubella RIA technique involved polyvinyl microtiter plates as the solid phase. When rubella virus was simply air dried in the wells of polyvinyl plates, there was a visible loss of antigen during washings. Fixation of the antigen by absolute methanol treatment for 5 min at room temperature reduced such losses. With fixed antigen, however, nonspecific adsorption of serum immunoglobulins was extensive and could not be reduced by treatment of the fixed antigen with blocking agents such as normal sheep serum, 1 or 4% BSA, or 0.1% gelatin (23). Such nonspecific adsorption of immunoglobulins resulted in high

background counts per minute, which made evaluation of test results difficult. Pretreatment of the plates with blocking agents before antigen fixation was also tested without beneficial results.

When rubella virus antigen was methanol fixed in wells of polystyrene microtiter plates, subsequent adsorption of nonspecific immunoglobulins was reduced approximately 80% compared with the polyvinyl plates. The disadvantage of using polystyrene plates as the RIA solid phase, however, was that the individual wells were difficult to cut out for radioactive counting. Preliminary tests were then conducted using more easily handled polystyrene balls, to which rubella antigen had been adsorbed. The RIA results obtained were indentical to those with the polystyrene plates; consequently, for practical purposes, polystyrene balls were chosen as the RIA solid phase.

At this point it is relevant to note that polystyrene balls have been used as the solid phase of a RIA method developed to detect hepatitis B antigen and antibody (M. Goldfield, personal communication). In addition, Abbott Laboratories (Chicago, Ill.) presently has available hepatitis B antigen and antibody RIA kits that also utilize polystyrene balls as the solid phase. When the polystyrene balls used in this study were compared with those kindly provided by Abbott Laboratories, no significant differences were noted.

Antigen adsorption onto polystyrene balls. Optimal RIA results were obtained when the polystyrene balls were incubated in a rubella virus antigen solution containing 110 μg of protein per ml (Fig. 1). Using an antigen concentration of 330 μg of protein per ml did not significantly enhance the specificity or sensitivity of the assays. With lower antigen concentrations of 27.5 or 6.9 μg of protein per ml, however, assay sensitivity was decreased. Methanol fixation of the antigen on the balls was also tried without obtaining any improvement in the RIA results. Altering the pH of the antigen diluent did have some effect, since using a pH of 8.35 or 9.0, rather than the usual 7.35, did increase the binding of specific immunoglobulins. Unfortunately, the binding of nonspecific immunoglobulins also increased in the same proportion.

When polystyrene balls were incubated at pH 7.35 in an antigen solution containing 110 μg of protein per ml, approximately 20% of the total protein became adsorbed. At the same time, the rubella hemagglutination titer of the antigen solution decreased between 70 and 80%, indicating that there was some preferential adsorption of the virus hemagglutinin. It was found

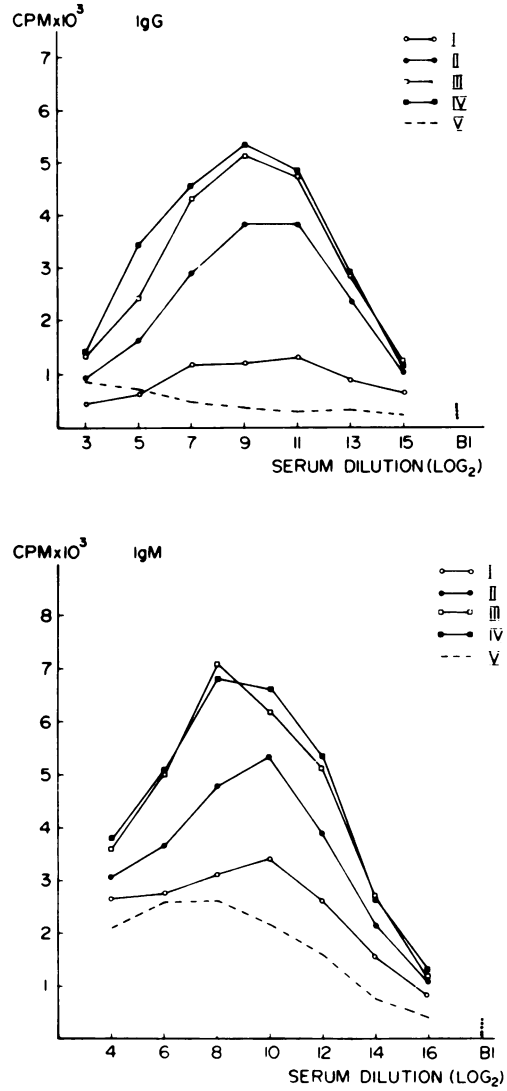

FIG. 1. *Effect of protein concentration on adsorption of purified rubella virus antigen on polystyrene balls. Balls adsorbed at each rubella virus protein concentration were incubated with dilutions of a rubella antibody-positive serum and tested for binding of rubella-specific IgG antibodies (upper) or IgM antibodies (lower). I, 6.9 μg of protein per ml; II, 27.5 μg of protein per ml; III, 110 μg of protein per ml; IV, 330 μg of protein per ml; V, 0 μg of protein per ml. In each panel, buffer blanks are designated as Bl.*

that an antigen solution could be used for adsorption only once, since the sensitivity of the assays decreased markedly after a second adsorption with fresh balls.

Incubation with serum dilutions. Best assay results were obtained when antigen-adsorbed balls were incubated with serum specimens

that had been diluted with 1% BSA-PBS, pH 7.35. Differences were actually found to be small, however, when the pH of the diluent was between 6.0 and 9.0. Various incubation times and temperatures were also tested. For both the IgG and IgM assays, increasing the incubation time or temperature resulted in greater binding of immunoglobulins from both rubella antibody-positive and -negative sera. For the IgG assay, the greatest difference between positive and negative sera was obtained by overnight incubation at room temperature. In the IgM assay, however, nonspecific immunoglobulin binding increased to such an extent with longer incubations that best results were obtained from a 1-h incubation at 37°C. For practical reasons, a 1-h incubation at 37°C was used for both assays.

Incubation with ^{125}I-labeled anti-human immunoglobulins. Dilutions containing 15,000, 30,000, 60,000, and 120,000 cpm/0.2 ml were tested. The binding ratios between rubella antibody-positive and -negative serum samples were almost identical in each case. The dilution containing 120,000 cpm/0.2 ml was used in the experiments in Fig. 1, whereas the dilution containing 30,000 cpm/0.2 ml was used throughout the remainder of this study. Dilution of the labeled anti-immunoglobulins with supplemented Eagle minimum essential medium (see Materials and Methods) was found to be essential, since background counts per minute levels increased greatly if the dilution was done with PBS. The effects of time and temperature on this step of the assays were not tested, since an incubation of 1 h at 37°C was found to be adequate.

Assay results. Background levels of the IgG and IgM assays were determined in three different ways. First, polystyrene balls with adsorbed control antigen (material obtained from supernatants of uninfected cells) were incubated with rubella antibody-positive and-negative control sera. Second, balls with adsorbed rubella antigen were incubated with negative control serum; and, third, balls with no adsorbed antigen were incubated with positive and negative control sera. In each case, the background binding levels of human IgG and IgM were reproducibly low. There was a marked difference, however, between the two assays. In the IgM assay, background levels were two to three times higher than in the IgG assay. Such higher nonspecific binding of IgM from serum samples, compared with that of IgG, has also been observed in other class-specific antibody RIA procedures (4).

Evaluation of assay results was done by means of binding ratios, which were calculated by dividing the counts per minute of the serum specimen by the counts per minute of the rubella antibody-negative control serum at the same dilution. The highest serum dilution having a binding ratio of 3.0 was chosen as the titer end point in the IgG assay. In the IgM assay, a binding ratio of 1.5 was selected with the proviso the counts per minute of the end point dilution also be 1.5 times the average counts per minute of all the negative control serum dilutions. End point titer values were obtained from the counts per minute versus the dilution curve of each serum specimen. Titer values are expressed as reciprocals, except in the figures and tables, where $\log_2$ values are used.

Intra-assay variation was studied by testing both a rubella antibody-positive and -negative control serum in 10 parallel determinations. The coefficients of variation of the counts per minute at each fourfold dilution were ±7.5% in the IgG assay and ±6.2% in the IgM assay. Variation in the assigned end point titer of a positive control serum was also tested and was found to be twofold in 15 separate IgG and IgM assays.

A comparison of the rubella HI antibody test and the rubella RIA IgG antibody test is shown in Table 1. The 32 serum specimens that were negative in the HI test were also negative in the RIA IgG test. Of the 19 specimens with an HI titer of 128 or less, 18 were highly positive in the RIA IgG test. One serum specimen having an HI titer of 8 was found to be negative in the RIA IgG test. The titer end points of the RIA IgG test were 16 to 256 times higher than the titer end points of the HI test, indicating that the RIA procedure is the more sensitive of the two techniques. The 51 serum specimens used in the experiments of Table 1 were also tested in the RIA IgM test, and all were found to be negative.

The development of rubella-specific RIA IgG and IgM antibodies in a series of serum speci-

TABLE 1. *Rubella HI and RIA IgG antibody titers of 51 serum specimens*[a]

HI titer ($\log_2$ value)	No. of serum specimens				
	<3[b]	9	10	11	12
<3	32				
3	1				
4		1	2		
5		4	3	3	1
6			2	1	
7					1

[a] The specimens were selected to represent 32 specimens with negative HI results and 19 specimens with relatively low HI titers.

[b] RIA IgG titer ($\log_2$ value).

mens collected from a rubella patient is shown in Fig. 2. The early rise and rapid fall of rubella-specific IgM antibodies and the slower rise of rubella-specific IgG antibodies are clearly demonstrated. A comparison of rubella HI, CF, RIA IgG, and RIA IgM antibody titers of 21 serum specimens collected from five rubella patients is shown in Table 2. The rapid development and early decline of IgM antibodies and the slower development of IgG antibodies is also apparent in these sera. Also demonstrated in Table 2 is the fact that both rubella class-specific antibody assays are much more sensitive than the rubella HI and CF tests.

DISCUSSION

The solid-phase RIA methodology described in the present study is both practical and reliable as a test for detecting rubella virus-specific antibodies in serum specimens. A distinct advantage of the procedure is that serum specimens can be tested without any pretreatment,

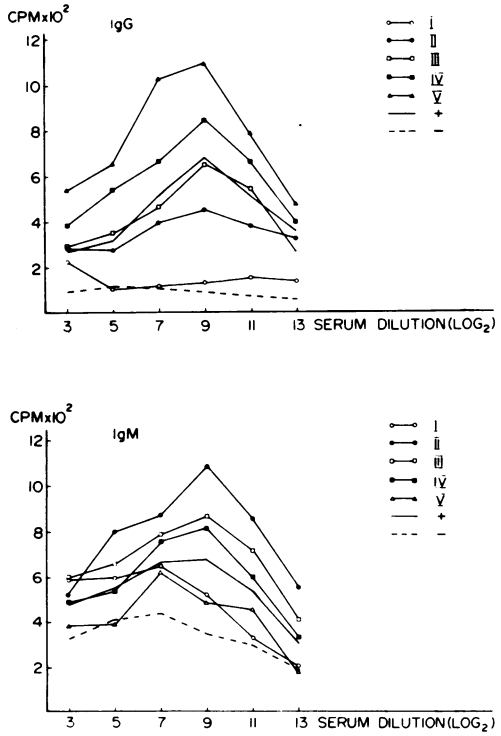

FIG. 2. *Development of rubella virus-specific RIA IgG antibodies (upper) and RIA IgM antibodies (lower) in a series of serum specimens from a patient with an acute clinical rubella infection. Serum specimens were obtained at: I, 3 days; II, 10 days; III, 17 days; IV, 24 days; V, 49 days, after onset of rash. (+) Rubella antibody-positive control serum; (–) rubella antibody-negative control serum.*

TABLE 2. *Rubella HI, CF, RIA IgM, and RIA IgG antibody titers in a series of serum specimens taken from five rubella patients*

Patient	Days after onset of rash	Titer (log_2 value)			
		HI	CF	RIA	
				IgM	IgG
V.V.	0	3	<2	3	3
	7	8	3	14	15
	14	8	4	14	15
	21	6	5	13	15
V.T.	0	<3	<2	<3	<3
	6	9	2	13	17
	12	8	3	13	17
	68	9	3	<3	17
V.S.	1	4	<2	13	<3
	12	9	4	16	14
	14	9	4	15	14
	21	9	4	15	14
S.S.	3	5	<2	6	7
	10	8	4	12	13
	21	9	4	12	13
	26	8	4	11	13
	37	9	4	<3	14
R.T.	1	6	<2	10	<3
	7	9	<2	14	15
	15	9	4	12	15
	26	9	4	10	15

since nonspecific inhibitors of the rubella hemagglutinin do not interfere. Once rubella virus-treated polystyrene balls and radioactively labeled antisera are available, the RIA method is simple to perform and specimens can be evaluated within 1 day. Since the assay results are recorded in printed form as counts per minute, there is no need for visual evaluation, as is the case with the rubella HI test.

Preliminary rubella RIA experiments were conducted with polyvinyl microtiter plates; this material, however, proved to be unsatisfactory as the RIA solid phase. When polystyrene balls were used, a marked improvement in the specificity and sensitivity of the rubella class-specific antibody assays was obtained. End point titers of positive sera in both the RIA IgG and the RIA IgM tests were very high (Tables 1 and 2), and in neither case were false-positive results seen in a test series of 77 specimens.

Positive-to-negative serum-binding ratios in the rubella RIA IgG test were consistently high, occasionally being 20 or more. Binding ratios in the RIA IgM test, however, rarely reached the level of 3.0. The difference between the two assays is due, first, to background levels being two to five times higher in the RIA IgM test and, second, to a slightly lower specific binding in the RIA IgM test compared with the RIA IgG test. In spite of these difficulties, the RIA IgM test clearly demonstrated the early

rise and rapid decline of rubella-specific IgM antibodies after onset of clinical rubella symptoms (Fig. 2, Table 2). The later appearance and subsequent persistence of rubella-specific IgG antibodies in the same serum specimens was also clearly demonstrated by the RIA IgG test.

In recent years, evidence has been accumulating that viruses may play a role in the etiology and/or pathogenesis of certain progressive neurological disorders (for a brief review, see reference 34). A sensitive and reliable assay is a necessary prerequisite for detection of low levels of antiviral antibodies that may be produced in the central nervous system. The rubella RIA IgG test would appear to fulfill these criteria, since it is 16 to 256 times more sensitive than the conventional rubella HI test (Table 1).

In summary, the RIA procedure described herein for the detection of rubella-specific IgG antibodies is feasible for clinical use. The same conclusion, however, cannot be drawn for the RIA detection of rubella-specific IgM antibodies. Although the results presented demonstrate that anti-rubella IgM antibodies can be measured with the described RIA methodology, further work is required to optimize the test conditions. In the present report, antibody levels detected by the RIA procedure are expressed in conventional serum titer values. By selecting a proper serum dilution and comparing its RIA counts per minute with a suitable standard curve, it may be possible to express the specific antibody content of a test serum in terms of micrograms per milliliter, as has been done in the case of chicken antibodies to BSA (33). Experiments to improve the rubella RIA IgM test and to quantitate both the IgG and IgM assays are currently in progress.

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