Solid-Phase Radioimmunoassay of Rubella Virus Immunoglobulin M Antibodies: Comparison with Sucrose Density Gradient Centrifugation Test

OLLI H. MEURMAN,* MATTI K. VILJANEN, AND KAISA GRANFORS

Departments of Virology and Medical Microbiology, University of Turku, SF-20520 Turku 52, Finland

Received for publication 8 October 1976

The solid-phase radioimmunoassay (RIA) method developed in our laboratory for demonstrating rubella virus-specific immunoglobulin G (IgG) antibodies (Kalimo et al., 1976) was further developed for demonstrating IgM antibodies. A total of 188 serum specimens were tested. The statistical probability of obtaining a false-positive IgM result, based on determinations of 100 rubella-negative sera, was below 0.001. Nonspecific inhibitors and IgM antibodies against other viruses tested did not interfere in the assay. In 2 out of 20 (10%) serum specimens with rheumatoid factor, a false-positive IgM result was obtained. The new RIA method was compared with sucrose density gradient centrifugation, followed by hemagglutination inhibition testing of the separated immunoglobulins with respect to demonstrating IgM antibodies. In patients with acute rubella infection, IgM antibodies were demonstrated by RIA in 9 out of 20 acute-phase sera and in all 20 early-convalescent-phase sera, compared with 7 out of 20 acute-phase sera and 19 out of 20 early-convalescent-phase sera by sucrose density gradient centrifugation. The results obtained indicate that the RIA method is reliable and sensitive and suitable for routine diagnostic use.

During the last few years, determination of specific immunoglobulin M (IgM) class antibodies for demonstrating a recent infection has become a widely accepted routine method in serological rubella diagnosis. Several techniques have been proposed, but the sucrose density gradient centrifugation method, originally reported by Vesikari and Vaheri (17) and Best et al. (2), has so far proved to be the most reliable and most commonly used (7).

In this report, the new solid-phase radioimmunoassay (RIA) method, described by Kalimo et al. (11) for demonstration of IgG-class rubella antibodies, has been further developed for measuring IgM-class antibodies. In addition, a series of experiments have been carried out to test the specificity of the IgM-RIA and to compare its sensitivity with that of the sucrose density gradient centrifugation method.

MATERIALS AND METHODS

Sera. A total of 188 sera from 118 patients were tested. These included 20 acute-phase and 20 early-convalescent-phase sera from 20 patients with acute rubella infection, 12 sera from patients with remote rubella infection, 16 sera from 10 patients with other acute virus infections, sera from 20 patients with rheumatoid factor (RF), and 100 sera from 62 patients with a negative rubella hemagglutination in-

hibition (HI) test. The sera were tested immediately or stored at -20°C until used.

RIA procedure. The methods described earlier (11) were used with the modifications indicated below. Briefly, rubella virus grown in suspension culture of BHK 21/13S cells was concentrated in an Amicon 402 Diaflo chamber containing an XM-300 membrane and purified by ultracentrifugation through 10% (instead of 20%) sucrose. The purified antigen was diluted in phosphate-buffered saline (PBS), pH 7.35, and adsorbed onto polystyrene balls by incubating balls submerged in an antigen solution containing 50 μg of protein/ml (as opposed to 110 μg of protein/ml [11]) at room temperature overnight. The antigen-coated balls were then incubated for 1 h at 37°C in 2% normal sheep serum, after which the balls were dried without washing. This treatment was added to the procedure to block additional nonspecific protein-binding sites on the balls surface. The balls were then incubated for 1 h at 37°C in fourfold serial dilutions of serum specimens. After washing, the balls were incubated at 37°C in a solution of ^{125}I -labeled anti-human-gamma or anti-human-mu immunoglobulin, with a specific activity of 10 to 20 $\mu\text{Ci}/\mu\text{g}$ (18). Thirty-thousand counts per minute (cpm) were added to each dilution tube in both the IgG and IgM assays. The incubation time was 1 h for anti-human-gamma, but overnight for anti-human-mu, because this was found to improve the sensitivity of the test. After washing, the balls were assayed for bound radioactivity in a gamma counter.

The RIA results are expressed as serum titers. In

calculating the end-point titers, only the linearly declining part of the cpm versus the serum dilution curve was used. The cutoff point used for positive specimens was three times the cpm of the negative control serum, with the proviso that the cpm of the test serum should also be 150 or more. The criteria for this cutoff point are given in Results. Before calculating the end-point titers, the cpm of buffer blanks were subtracted from the cpm values of the test serum and negative control serum. Using the cutoff point, the end-point titer was then read from the dilution scale as the reciprocal of the nearest twofold serum dilution.

Sucrose density gradient centrifugation. The method of Forghani et al. (7) was used. Stepwise gradients were prepared in 5-ml tubes by layering 0.9-ml volumes of 26% (wt/wt), 22%, 18%, 14%, and 10% sucrose solutions diluted in PBS, pH 7.4, from bottom to top. The gradients were equilibrated by diffusion at 4°C for 24 h, and 0.5 ml of test serum diluted 1:2 in PBS was layered on top of the gradient with slight mixing. The tubes were centrifuged at $135,000 \times g$ for 16 h in a Spinco SW39 or SW50.1 rotor. The first 1.5 ml from the bottom was collected as the IgM fraction, the next 0.8 ml was discarded, and the next 1.5 ml was collected as the IgG fraction.

Immunodiffusion tests. Gradient fractions were tested against anti-human-IgM (mu-chain-specific) and anti-human-IgG (gamma-chain-specific) reagents (Burroughs Wellcome Ltd, Beckenham, Kent, England) by immunodiffusion tests using a 1% agarose gel on a glass slide. The slides were incubated in a moist chamber for 48 h at room temperature, after which the precipitation lines were read. If contamination of fractions was noted, the centrifugation procedure was repeated.

Other serological tests. Assay for rubella HI antibody was conducted by the Center for Disease Control Modified rubella HI test (16). Nonspecific inhibitors were removed from the gradient fractions with soluble phospholipase-C as described by Haukenes and Blom (10). Complement fixation (CF) tests were performed with the standardized microtechnique (3). IgM antibodies to Epstein-Barr virus (EBV) were demonstrated by indirect immunofluorescence technique (IFAT) (13), and to measles and herpes simplex viruses by RIA tests (1, 12). RF was removed from the serum specimens by absorption with heat-aggregated human IgG (15).

RESULTS

Estimation of cutoff point for determination of end-point titers. The actual cpm levels in the tests varied somewhat from day to day when different antigen and/or anti-human-immunoglobulin lots were used. Also, with the aging of the labeled anti-human-immunoglobulin its activity and the mean cpm levels in the tests decreased. For these reasons, it proved to be most reliable to determine the cutoff point in relation to a standard negative control serum, which was the same in every test series. The cpm values of the negative control serum

FIG. 1. Distribution of binding ratios (cpm of test serum/cpm of negative control serum at the same dilution) of 100 rubella negative sera in IgM assay (upper) and IgG assay (lower). Mean $\pm$ standard deviation as well as highest and lowest value obtained are given.

were, however, sometimes very low or even unmeasurable, and therefore a corrected minimum value of 50 cpm was established for calculation purposes. Figure 1 shows the distribution of binding ratios (cpm of the test serum per cpm of the negative control serum at the same dilution) of 100 rubella-negative sera in IgG and IgM assays. The mean level of binding ratios in both assays was close to 1.0, with the exception of the higher serum dilutions in the IgG assay, where the difference is caused by the corrected minimum value of the negative control serum. This indicates that the negative control serum used was a good representative of rubella-negative sera. The highest binding ratios of the negative sera were 2.98 in the IgG assay and 2.50 in the IgM assay. The ratio 3.0 was chosen for the cutoff point, and because the negative control serum had a minimum value of 50 cpm,

this led to the proviso that the cpm of the test serum had to exceed 150. With these criteria and on the presumption that the scatter of the binding ratios follows the normal distribution, the probability of obtaining a false-positive result is less than 0.001 for the IgM assay and 0.003 for the IgG assay.

Tests for specificity. The effect of nonspecific inhibitors on the RIA technique was tested by fractionating some sera in sucrose density gradients, as described in Materials and Methods, after which 10 0.5-ml fractions were collected dropwise through the bottom of the tube. The fractions were tested by HI, IgG-RIA, and IgM-RIA. Table 1 shows the results obtained with three representative sera. In the HI results, three peaks corresponding to IgM, IgG, and nonspecific inhibitors were observed, whereas in the RIA results, the inhibitor containing fractions remained negative.

Sixteen sera from patients with acute viral infections other than rubella, including EBV, measles, herpes simplex, mumps, adeno- and enterovirus infections, were tested for rubella-IgM antibodies. The presence of the respective specific IgM antibodies was demonstrated in EBV, measles, and herpes simplex infections as described in Materials and Methods, whereas in the case of mumps, adeno-, and enterovirus infections it was assumed from the demonstration of an acute infection by a fourfold or greater rise in the CF titers between acute-phase serum and early-convalescent-phase serum taken 10 to 14 days after the acute-phase serum. All sera were negative for rubella IgM antibodies, which confirms that other anti-viral IgM antibodies do not interfere in the RIA.

Sera from 20 patients with RF were tested by

RIA for IgM antibodies. Two of these were positive. One serum taken from a 11-year-old boy with systemic lupus erythematosus had a titer of 128,000, another serum taken from a 63-year-old male with rheumatoid arthritis had a titer of 1,028. After absorption with aggregated human IgG the latter serum became negative, whereas the titer of the former serum did not decrease. This serum was positive in the IgG-RIA but negative in the HI test, and the patient had not had a rubella infection anamnistically. Also, in the case of measles IgM-RIA this serum gave a positive IgM result, and both the IgM results were considered to be false positive. Six sera that were negative in the IgM-RIA were also tested for EBV IgM antibodies by the IFAT. In this test they all gave a false-positive IgM result.

Comparative sensitivity of sucrose density gradient centrifugation and RIA. Table 2 shows the efficacy of sucrose density gradient centrifugation followed by HI test and of RIA

TABLE 2. Comparative efficacy of sucrose density gradient centrifugation followed by HI and RIA tests for detection of rubella IgM antibodies

Time of specimen collection (days after onset)	No. of sera tested	No. of serum specimens with IgM antibodies demonstrable by:		
		Density gradient centrifugation + HI	IgM-RIA of whole serum	Density gradient centrifugation + IgM-RIA
0-2	8	0	1	0
3-7	12	7	8	8
8-24	20	19	20	19
Remote infection	12	0	0	Not tested

TABLE 1. Rubella HI, RIA-IgG, and RIA-IgM antibody titers of serum fractions after sucrose density gradient centrifugation

Fraction no. ^a	Titer								
	Negative serum			Acute-phase serum			Convalescent-phase serum		
	HI	RIA		HI	RIA		HI	RIA	
		IgG	IgM		IgG	IgM		IgG	IgM
1	<1	<1	<1	8	<1	2	<1	<1	<1
2	<1	<1	<1	8	<1	16	<1	<1	<1
3	<1	<1	<1	8	<1	4	<1	<1	<1
4	<1	<1	<1	4	4	<1	1	16	<1
5	<1	<1	<1	8	32	<1	4	32	<1
6	<1	<1	<1	8	64	<1	4	64	<1
7	<1	<1	<1	8	64	<1	8	64	<1
8	1	<1	<1	8	16	<1	32	8	<1
9	4	<1	<1	16	4	<1	32	<1	<1
10	8	<1	<1	16	2	<1	32	<1	<1

^a From bottom to top.

tests, in demonstrating rubella IgM antibodies in 52 serum specimens from patients with post-natal rubella infection. The RIA test was more effective; in three cases IgM antibodies could be demonstrated by RIA, but not with HI test after sucrose density gradient fractionation. No increase in the efficacy of the RIA test could be gained by fractionation of the sera before testing. None of the sera from patients with remote rubella infections showed IgM antibodies by any of the methods.

The comparison of rubella IgM titers obtained by these methods is demonstrated in Fig. 2. The correlation between HI titers and RIA titers is rather good, RIA titers being 8 to 256 times higher than HI titers, when fractions positive by both methods are compared. The correlation between rubella IgG titers is demonstrated in Fig. 3. In 11 cases the HI titer became negative after removal of nonspecific inhibitors with phospholipase-C. Seven of those sera were also negative on being tested when unfractionated by HI test, whereas the remaining four sera had low HI titers of 8 to 32. It is obvious that the very high HI titers of these fractions were caused by the trailing of nonspecific inhibitors into the IgG fractions.

DISCUSSION

The RIA method described here is highly specific and sensitive. With two major modifica-

FIG. 2. Comparison of rubella IgM titers obtained by sucrose density gradient centrifugation followed by HI and by RIA tests.

FIG. 3. Comparison of rubella IgG titers obtained by sucrose density gradient centrifugation followed by HI and by RIA test. An open circle indicates HI titer which became negative after removal of nonspecific inhibitors with phospholipase-C.

tions: pretreatment of the balls with sheep serum to decrease the nonspecific binding of proteins on the ball surface and lengthening the incubation time of the antihuman-mu-immunoglobulin, the sensitivity of the IgM assay improved decisively. Whereas the binding ratios obtained in the previous studies (11) rarely exceeded 3.0, they were now often between 10 and 20.

The most serious source of error in the HI test is the nonspecific inhibitors, which should be totally removed from serum before testing. Unfortunately, none of the routine methods is fully satisfactory (10, 14). In consideration of this, it is a great advantage that the nonspecific inhibitors do not interfere in the RIA test, and serum specimens can be tested without any pretreatment.

RF, which is an anti-IgG immunoglobulin of the IgM class, is known to cause false-positive IgM results in IFAT (6). In the RIA method, where the principle of the test is analogous to that of the IFAT, false-positive IgM results were also expected. Unexpectedly, only 2 out of 20 pa-

tients with RF gave a false-positive IgM result in the RIA test. The reason for this is unknown, but a speculative explanation can be given.

It has been shown that RF has a very much greater affinity to aggregated than to unaggregated IgG (5), a phenomenon that is due to polyvalent interactions between antibody and antigen molecules (4). In IFAT, where the antibodies attach to the intracytoplasmic antigen in fixed cells, there will be a large number of antigenic deposits in close proximity available. Such situations may be favorable for the formation of antibody aggregates. The granular appearance of cytoplasmic fluorescence also supports this theory. In RIA, on the other hand, the viral antigen is rather thinly settled on the ball surface (K. Kalimo, unpublished scanning electron microscopy observations), and analogous possibilities of aggregate formation are absent. This might explain the low affinity of RF to the attached IgG antibodies in the RIA system. The study material here is, however, small, and the role of RF in the IgM-RIA needs further investigation with larger numbers of sera.

If a positive IgM result in RF-containing serum is suspected to be a secondary one caused by RF, either this or IgG immunoglobulins have to be removed from the specimen before retesting. The RF can usually be removed by adsorption with aggregated IgG (15) or with kaolin (9), whereas in sera with very high RF content, a more efficient method such as immunoabsorption with anti- γ Fc can be used (8).

The reason for positive rubella IgM results in the serum of the boy with systemic lupus erythematosus remains unclear. The results can be explained by the binding of RF to rubella antibodies of the IgG class, if the adsorption with aggregated human IgG was not able to remove sufficient amount of RF activity from the serum. On the other hand, besides the RF there were also other immunological anomalies present, e.g., IgA deficiency and high titers of antinuclear antibodies of both the IgG and IgM class. Thus, the false-positive IgM result might be caused by some immunological factor independent of RF. This could also explain why the adsorption of RF with aggregated IgG did not lead to a decrease in the IgM titer.

The IgM-RIA, as described, is more sensitive than the sucrose density gradient centrifugation test. This can be of advantage in special conditions, but for routine diagnostic purposes the sensitivity of both methods is quite satisfactory. Both methods require special laboratory equipment, but the RIA test is technically much easier and a larger number of serum specimens can be handled per day. The need for purified viral antigen and specific anti-human-

immunoglobulins is a limitation to the method, but apparently these will be obtainable commercially in the future. The reliability of the test, with the statistical probability of a false-positive result below 0.001 and 0.003 for the IgM and for the IgG assay respectively, is extremely good among serological techniques. The RIA test possesses all the prerequisites to be adopted as a standard diagnostic method in central virus diagnostic laboratories.

ACKNOWLEDGMENTS

This study was supported by grants from the State Medical Research Council of Finland and the Sigrid Juselius Foundation.

The excellent technical assistance of Leena Soini and Soile Niittoaho is gratefully acknowledged.

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