Single Radial Hemolysis Test for Rubella Immunity and Recent Infection

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The single radial hemolysis (SRH) test was compared with the hemagglutination inhibition (HI) test for establishing rubella immune status and diagnosing recent infection. Correlation between mean SRH diameters and HI titers \( \geq 1:8 \) was high \((R = 0.99)\). It is suggested that a level of \( \geq 5\) IU represents low-level antibody and that \( \geq 15\) IU is a conservative threshold for designation of immunity. Of 343 sera tested, only 1 false-positive was found by SRH with the 5 IU cutoff level. The SRH test could detect serum antibody levels as low as 2.5 IU, whereas 15 IU was generally the limit of resolution of the HI test. Data from sucrose density gradient fractionation of serum demonstrated that neither rubella-specific immunoglobulin M (IgM) nor early postinfection HI-reactive IgG was detected by SRH. However, diagnostic changes in antibody titer measured by SRH were in general greater than those measured by HI. The SRH test showed diagnostic titer changes in some sera containing specific IgM for which no such changes were detected by the HI test. A 2.5-mm difference in hemolytic zone diameters (a fourfold rise in international units) between acute- and convalescent-phase serum pairs was chosen as being of diagnostic significance. This difference was less than the minimum observed difference of 2.9 mm from 59 serum pairs showing diagnostic changes by HI and far exceeded \((>3.6\) standard deviations\) the within-test and individual variability seen for 95 pregnant women from whom samples were obtained during each trimester.

In single radial hemolysis (SRH) tests, test sera are added to wells in an agarose gel containing antigen-sensitized erythrocytes. In the presence of complement, sera produce areas of hemolysis proportional to the concentration of specific antibody. As an alternative to the hemagglutination inhibition (HI) method for rubella antibody determination, SRH has the advantage that no pretreatment of sera for removal of nonspecific inhibitors and agglutinins is required. Moreover, in the SRH test only small volumes of sera are required (5 \( \mu l \)), and the test is simple to perform, easy to read, and relatively inexpensive (6).

The SRH test has gained wide acceptance in a number of countries as a screening test for rubella antibodies, although it is not widely used for this purpose in North America. The British Public Health Laboratory Service and other laboratories in Sweden and Australia have adopted the SRH test for immune status determination (9, 11, 20). Mortimer et al. (13) suggested recently that the threshold for reporting immunity to rubella be lowered from 15 IU of antibody per ml. Individuals having some detectable antibody at levels below 15 IU fail to produce rubella-specific immunoglobulin M (IgM) antibody in response to vaccination with RA 27/3; however, individuals without detectable SRH antibody do (13). The question of what constitutes a diagnostically significant difference between the SRH antibody levels in acute- and convalescent-phase sera has been addressed by few authors. Mortimer et al. (13) consider an increase in zone diameter of \( \geq 4 \) mm between serum pairs as a significant rise. Skaug et al. (18) chose 1 mm (2 standard deviations [SD]) as a significant difference in hemolytic zones between paired sera. Forger and Gilfillan (6) used a fourfold HI equivalent change in SRH titer for diagnosis of recent rubella infection. Antibody of the IgM class is not detected by SRH (9, 16, 20). Champsaur et al. (4) reported that SRH does not detect antibody earlier than 6 days after the onset of rash.

In this study data are presented to show that selecting an SRH diagnostic diameter difference equivalent to a fourfold change in international units yields a diagnostic index which is both sensitive and highly specific due to the small degree of variability that occurs in the SRH test. The threshold SRH antibody level indicative of immunity is discussed.
Data from sucrose density gradient fractionation of sera confirm that rubella-specific IgM antibody is not detected by SRH, and in addition demonstrate directly that early postinfection IgG antibody is not detected by SRH.

MATERIALS AND METHODS

SRH test. Sheep erythrocytes stored in Alsever solution (Institut Armand-Frappier, Laval, Quebec) for 4 days to 3 weeks were washed three times in phosphate-buffered saline and adjusted to 20% in Veronal buffer; both buffers were at pH 7.2. Equal volumes of hemagglutinating antigen (Flow Laboratories, McLean, Va.) with a minimum titer of 1:128 and 20% erythrocytes were mixed and incubated at 4°C for 30 min. These antigen-sensitized cells were washed three times in Veronal buffer and adjusted to 10%. Agarose (Agarose II; Sigma Chemical Co., St. Louis, Mo.) was brought to 1% in Veronal buffer and autoclaved for 10 min at 115°C, and then 1.5 ml of sensitized erythrocytes, 0.5 ml of guinea pig complement with a minimum activity of 280 50% hemolytic units per ml, and 13 ml of 1% agarose, all at 45°C, were mixed and immediately poured into 9- by 9-cm petri plates (Lab-Tek Products, Miles Laboratories, Naperville, Ill.). Wells (3 mm diameter, 36 per plate) were punched into the hardened agarose, and 5 μl of heat-inactivated serum was added to each well. Control plates (for nonspecific hemolysis) were processed in the same manner, with Veronal buffer substituting for hemagglutinating antigen. Plates were incubated in a humidified incubator at 37°C for 18 h. Limits of zones of hemolysis were marked with needle pricks on bottoms of plates, and diameters were measured with a graduated magnifier (Hyland Diagnostics, Deerfield, Ill.) to the nearest 0.1 mm. Prepared plates, when stored at 4°C, were usable for up to 2 weeks before serum addition without loss of resolution of the zones.

Serum inactivation. The effect of serum inactivation on SRH patterns was examined. Sera were inactivated at 56°C for 0, 15, 45, or 60 min or at 60°C for 20 min.

Standard curve. For each test, a standard curve was constructed from reference serum dilutions to convert zone diameters to international units. Dilutions were made from the World Health Organization (W.H.O.) international anti-rubella serum, second reference preparation, 1970 (Statens Seruminstitut, Copenhagen, Denmark). Dilutions with 500, 100, 50, 25, 15, and 5 IU of antibody were used for the standard curve.

Other antibody test methods. The HI method of Halonen et al. (8) with kaolin serum treatment at pH 9.0 and chick indicator cells was used, modified only in that cold incubation was at 4°C. Rubazyme enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, North Chicago, Ill), Ortho Rubella ELISA (Ortho Diagnostics, Don Mills, Ontario), and Gamma Coat radioimmunoassay (RIA) (Clinical Assays Division of Travenol Laboratories Inc., Cambridge, Mass.) tests were conducted on those sera which gave discrepant results by HI and SRH. In sera fractionated by sucrose density gradient (SDG) centrifugation (14), the presence of rubella-specific antibodies was detected by HI testing of fractions. An 18-h incubation of antigen and antibody was used to enhance sensitivity. Immunoglobins in the fractions were identified by class-specific radial immunodiffusion plates for low levels of human immunoglobulins (Hyland Diagnostics, Deerfield, Ill.). The presence of rubella-specific antibodies in the IgM fractions was confirmed by a fourfold reduction of HI titer by 2-mercaptoethanol treatment (15).

Sera. Three hundred and forty-three single serum specimens used in this study were received for rubella immune status determination. Serum samples taken from patients during each trimester of pregnancy and immediately postpartum were provided through the cooperation of Juan Embil of the Infectious Disease Research Laboratory, Izaak Walton Killam Hospital for Children, Halifax, Nova Scotia. Acute- and convalescent-phase serum pairs were submitted for reference testing or provided by Micheline Fauvel, Institut Armand-Frappier, Laval, Quebec. Rheumatoid factor (RF)-positive sera, as determined by RA Test (Hyland Diagnostic), were provided by J. Dunne, Rheumatology Department, Ottawa General Hospital, Ottawa, Ontario. Sera containing high levels of heterophile antibody were supplied by J. H. Joncas, Microbiology Department, St. Justine Hospital, Montreal, Quebec.

RESULTS

Serum inactivation. The effect of serum inactivation on hemolysis patterns was investigated. Most non-inactivated sera produced faint hemolysis patterns. Similarly, most non-inactivated sera had halos of incomplete lysis surrounded by an area of greater lysis. Zones exceeding 5-mm diameter in 15 min were much clearer and improved further with increased inactivation time. A 1-h period of inactivation at 56°C did not give any improvement in results over a 45-min period of inactivation. Inactivation at 60°C for 20 min produced the clearest zones with the fewest halos, and this inactivation period was adopted as standard procedure throughout the study.

NSH. The observed incidence of nonspecific hemolysis (NSH) in control plates was 2.3%, with NSH exceeding a 5-mm diameter in 0.5% of the sera tested. The minimum observed difference between NSH and rubella-specific diameters was 3.4 mm. Some NSH may be attributable to heterophile antibody. In 10 sera containing high levels of heterophile antibody, 4 produced NSH zones >3.5 mm. These zones were abolished by sheep erythrocyte absorption.

Halo formation. The association of RF-containing sera and anticomplementary (AC) sera with halo formation was examined. For 50 sera containing RF the proportion of sera showing halos was 12%, compared with 16% for non-RF sera. To determine whether the halos might be due to AC activity, we examined 25 sera showing AC activity at 1:8 or greater in complement fixation tests. The degree of halo formation (20%) was not significantly greater than it was in non-AC sera (16%).

Comparison of SRH and HI titers. The relationship between SRH diameters and HI titers is
shown in Fig. 1. The correlation coefficient between HI and SRH titers was 0.84. Correlation between mean SRH diameters and HI titers >1:8 was 0.99. Among the sera tested, there were almost as many sera with HI titers of 1:8 (18.3%) as with titers <1:8 (19.0%). The frequency of remaining HI titers was 26.8, 21.3, and 13.2% for titers of 1:16, 1:32, and 1:64, respectively. Figure 2 shows the frequency distribution of SRH titers in the population sampled. Relatively few sera had titers at the 5 to 15 IU level. The modal titer was 100 IU (11.5 mm diameter).

Immune status determination. Generally, HI titers >1:8 and SRH titers >15 IU (11) have been considered as indicating immunity. Sera (Fig. 1) with titers <1:8 by HI or <15 IU by SRH were examined further. With 15 IU used as an indicator of immunity, two sera with HI titers of <1:8 contained >15 IU by SRH. Both sera showed an antibody level of 1:4 by HI. The four sera with a titer of 1:8 by HI but containing <15 IU by SRH were shown to have low levels of antibody by ELISA and RIA. Four sera with HI titers <1:8 contained between 5 and 15 IU by SRH; three of these sera were positive by ELISA and RIA. The remaining serum was negative by ELISA and RIA.

Within-test variability. Within-test variability for SRH was studied by randomly assigning each of 20 sera to five different wells. The largest range of SRH diameters among the five replicates of 20 sera was 1.3 mm (13.4 to 14.7 mm). The mean SD was 0.33 mm, and the largest SD was 0.52 mm. The mean coefficient of variation among replicate sera was 3.4%.

Individual variability. For 95 sets of four serial samples from pregnant women, the corresponding values for largest range, mean SD, and largest SD were 1.7, 0.39, and 0.69 mm, respectively. These variations reflect both within-test and individual variation over time. If the 0.69-mm SD is used as an indication of maximum variability, the probability of a 2.5-mm or greater difference in diameters between two sera being due to chance or normal individual variation is <0.0001 (3.6 SD). A 2.5-mm change in diameters corresponds to a fourfold difference in international units. Although no diagnostic differences by HI tests were seen among the sets of serum samples taken during pregnancy, changes in SRH antibody level up to 58% could be detected between the first and third trimester. Of the 95 sets of sera, 81 (85.3%) showed a decrease in antibody titer and 14 (14.7%) showed an increase. The mean net change in
HEMOLYSIS TEST FOR RUBELLA

Frequency distribution of 343 sera with rubella antibody titers expressed in SRH diameters and international units.

The titer was an 18% decrease. No change was seen between first and third trimester samples by the HI test in 77.4% of the pregnancies; a twofold decrease was seen in 19.4%, and a twofold increase was found in 3.2%. The decrease in IgG levels showed similar variability to the SRH titers, with an overall decrease in total IgG of 18.6% between first and third trimester, as measured by immunodiffusion plates.

Sensitivity of SRH. With serially diluted W.H.O. reference serum, SRH could detect 2.5 IU of antibody (~4 mm diameter), whereas the HI test detected no less than 15 IU. By adding 20 µl of serum and allowing diffusion for 24 h (5), we detected 1.25 IU of antibody (~4 mm diameter).

Acute- and convalescent-phase serum pairs. The data in Table 1 show the mean titers and minimum titer differences for HI and SRH tests on 59 acute- and convalescent-phase serum pairs. The minimum observed difference by SRH between serum pairs was 2.9 mm (4.3-fold increase in international units).

Five additional pairs of acute- and convalescent-phase sera which gave identical titers by the HI test showed significant differences (four-fold rise in international units) by SRH. The acute serum of each pair contained specific IgM antibody.

**TABLE 1.** Comparison of SRH and HI titers for 59 acute- and convalescent-phase serum pairs showing at least fourfold titer changes by HI

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean titer</th>
<th>Minimum titer difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Convalescent</td>
</tr>
<tr>
<td>HI</td>
<td>6.5</td>
<td>166.2</td>
</tr>
<tr>
<td>SRH diam (mm)</td>
<td>3.9</td>
<td>13.2</td>
</tr>
<tr>
<td>SRH titer (IU)*</td>
<td>1.9</td>
<td>219.0</td>
</tr>
</tbody>
</table>

* Obtained from standard curve.

**FIG. 2.** Frequency distribution of 343 sera with rubella antibody titers expressed in SRH diameters and international units.

**IgM-containing sera.** SRH results on 23 sera containing rubella-specific IgM are summarized in Table 2. Of these sera, 17 showed significant titers by SRH (>15 IU). One serum which failed to show any hemolytic activity contained only IgM antibody. The remaining five sera were collected within 4 days of onset of rash and contained IgG as well as IgM antibody. Two of these five sera were negative by SRH, but three produced inconsistent lysis, i.e., very faint zones of hemolysis were seen in one of three tests. No specific hemolytic activity was found in any of the IgM fractions. The three unfractionated sera which were consistently negative by SRH also failed to demonstrate specific hemolytic activity in the IgG fractions.

**TABLE 2.** Comparison of SRH and HI titers for 23 acute- and convalescent-phase serum pairs showing at least fourfold titer changes by HI

<table>
<thead>
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* Obtained from standard curve.
TABLE 2. Effect of rubella-specific IgM and early IgG on SRH

<table>
<thead>
<tr>
<th>No. of sera</th>
<th>Sera obtained after onset of rash (days):</th>
<th>Presence of specific:</th>
<th>SRH result</th>
<th>Equivocal</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>IgG</td>
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<td>17</td>
<td>&gt;6</td>
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<td>17</td>
</tr>
<tr>
<td>1</td>
<td>&lt;4</td>
<td>Yes</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>&lt;4</td>
<td>Yes</td>
<td>Yes</td>
<td>2</td>
</tr>
</tbody>
</table>

a Obtained from HI tests on sucrose density gradient fractions. Antibody class was identified by immunodiffusion plates. Specific IgM was confirmed by 2-mercaptoethanol reduction of HI titer.
b Diameter of lysis >15 IU, equivalent to W.H.O. international anti-rubella reference serum.
c No specific hemolysis.

DISCUSSION

Kurtz et al. (11) found that serum inactivation at 60°C for 20 min reduced the frequency of NSH in SRH to below that observed with the usual 56°C for 30 min regime. In this study, 60°C inactivation optimally reduced the incidence of halo formation and produced the clearest zones of lysis. Gee et al. (7) reported that RF may interfere with SRH. In this study the incidence of halo formation was no greater with RF-positive than with RF-negative sera. Similarly, AC sera did not significantly increase halo formation.

NSH did not interfere with the interpretation of any serum titer. Should NSH interfere with test reading and erythrocyte adsorption fail to remove NSH, an alternative test method should be used.

The linear relationship between HI titer and SRH diameter for sera with HI titers >1:8 (Fig. 1) confirmed that found by other authors (7, 9, 11, 17). With 15 IU used as the cutoff, two sera were falsely positive relative to the HI titer of 1:8, but these sera had HI titers of 1:4 and were also RIA and ELISA positive, and were therefore considered to be true antibody positives. Seven sera identified as antibody positive by all other test methods would be designated SRH negative at the 15 IU level; the SRH result would be in agreement with other test methods if a 5 IU cutoff were used. However, one serum which appeared to contain about 10 IU of antibody was negative by HI (1:4), RIA, and ELISA and judged a true false-positive result if 5 IU is taken as the level indicative of rubella immunity. This serum produced a very faint zone of lysis in SRH. When sera from non-pregnant women are being tested to determine the need for vaccination false-positives are unacceptable, and a more conservative antibody level (15 IU) might be used to indicate immunity; those with titers below this level would be recommended for vaccination. For sera from pregnant women where false-negative findings of immunity would also cause concern, a lower cutoff level of 5 IU could be considered, and the status of an individual with antibody >5 but <15 IU could be regarded as probably immune or at low risk but subject to serological monitoring during the pregnancy. Absence of an IgM response to vaccination in individuals with rubella antibody at this level suggests that 5 IU may indicate immunity (13).

False-positive antibody detection by conventional tests ranges from 3 to 14.3% (1, 2, 12, 19, 21). False positivity in assays by commercial kits may reach 27% (3). In light of these unacceptable levels, the highly specific SRH may be an attractive alternative for immune status determination, despite the requirement for biweekly plate preparation.

That the SRH test can be made extremely sensitive was shown by using the modification of Champsaux and Slim (5), which allowed detection of 1.25 IU with a larger inoculum and a longer diffusion period. Even without this modification, the SRH test detected 5 IU of antibody, well below the 15 IU detectable by the HI test.

The distribution of SRH diameters (Fig. 2) showed that very few sera had antibody levels in the critical region within which a decision about immune status is made (5 to 15 IU). With the HI test, there were about as many sera with titers of 1:8 as with titers <1:8; many more sera fell within the borderline immune-susceptible region.

Rubella SRH does not detect IgM, nor does it reliably detect very early IgG (<5 days post-rash), although with some sera early IgG may produce faint lysis (Table 2). Hoppe and Drescher (10) indicated that early antibody to rubella is of a different quality than later antibody. The early IgG antibody may lack the ability to activate or bind complement as effectively as later antibody does. The insensitivity of SRH to IgM and early IgG may be used to advantage in that serum pairs taken too late to show a fourfold rise in HI titer may still demonstrate a diagnostic increase by SRH; indeed, five pairs of sera showed such an increase by SRH. Although specific IgM was present in each case,
no evidence of recent infection was seen based on HI titers alone. This emphasizes the utility of alternative methods which detect later-rising antibody when exposure to rubella is suspected.

A 2.5-mm difference in SRH diameters between serum pairs was chosen as being of diagnostic significance. The rationale for using the 2.5-mm difference was threefold. (i) By convention, a fourfold rise in serological tests is diagnostically significant, and a fourfold change in international units measured by SRH corresponds to ~2.5 mm. From the slopes of standard curves prepared for each SRH test, a fourfold change in international units corresponds to a diameter change of 2.5 mm. (ii) This difference in diameters is less than the minimum observed difference of 2.9 mm between 59 acute- and convalescent-phase serum pairs with demonstrable fourfold changes in HI titer. For the most part, the diameter differences were dramatic (mean difference, 9.3 mm). (iii) A 2.5-mm change (fourfold change in international units) far exceeds the combined within-test and individual variability (largest SD, 0.69 mm) seen in the sequential samples from 95 pregnant women. The within-test variability of the SRH test for replicates of the same serum was small (mean SD, 0.33 mm). However, this does not take into account possible inherent fluctuations in antibody level in the same individual over time. The variation in antibody level between first trimester and term in the sera of 95 pregnant women was also small (mean SD, 0.39 mm). From the foregoing discussion, an SRH diagnostic diameter difference based on a fourfold change in antibody level measured in international units appears to be a reasonable, although conservative, choice. With further clinical experience and study, less than fourfold changes in antibody level may be found to be an acceptable diagnostic criterion for tests with high levels of precision.

In this study, the 18% decrease in rubella SRH titer between first and third trimester in all cases represented a less than fourfold change in HI titer. This decrease in specific antibody was consistent with the observed overall decrease in total IgG of 18.6%. In the population examined by Skaug et al. (18), a mean decrease in rubella SRH titer of 30% was reported between the 13th and 35th week of gestation. The decrease in antibody levels may be attributable to an increased plasma volume or hemodilution during pregnancy (22).

In summary, the SRH test provides a specificity and sensitivity which make it suitable for diagnosis of recent infection as well as for immune status determination. Interlaboratory variability in the reporting of immune status or diagnostic changes may be reduced by use of an international standard serum (or secondary national reference preparations) with SRH titers expressed in international units.

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LITERATURE CITED


