Nonspecific Reactions in the Hemagglutination Inhibition Test for Detection of Rubella Antibodies

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Received 18 August 1980/ Accepted 4 February 1981

Approximately 7% of the sera tested to determine the presence of rubella-specific antibodies by the hemagglutination inhibition test demonstrated abnormal patterns of reactivity, rendering the test unreadable. Another 3% of sera were shown to have false-positive titers as high as 1:128. When these abnormally reacting and false-positive sera were heated at 56°C for 30 min after chemical treatment they always converted to negative, indicating the absence of specific rubella hemagglutination-inhibiting antibody. These results were confirmed by fractionation of the sera after sucrose gradient centrifugation. It was established that manifestation of these nonspecific results was dependent on the concentration of Ca²⁺ or Mn²⁺. The heat-labile inhibitor(s) responsible for abnormal and false-positive reactions was found not to be complement. This inhibitor(s) was detected in the light fractions of sera and when added to negative sera was capable of reproducing the abnormal patterns of reactivity. These results emphasize the necessity of heating sera for the rubella hemagglutination inhibition test after the chemical removal of nonspecific inhibitors.

The hemagglutination inhibition (HI) test for the detection of rubella antibodies developed by Stewart et al. (12) is the most widely used test for both the serological diagnosis of rubella and for the determination of immune status. Non-immunoglobulin inhibitors of the rubella hemagglutination reaction which are present in human sera interfere with the detection of specific rubella antibodies and, if not completely removed, can cause a false-positive result. Two methods commonly used to remove these inhibitors involve the treatment of sera with either heparin-MnCl₂ or dextran sulfate-CaCl₂ (10). We have found that these standardized procedures may yield a certain percentage of results which are either difficult to interpret or false-positive. The present report deals with this problem and its possible causes and presents a simple modification of the rubella HI test which eliminates the problem.

MATERIALS AND METHODS

Sera. The sera used in this study were human specimens sent to the laboratory for titration of rubella antibody. Sera were stored for periods of up to 3 months at −20°C until tested. They were received by the laboratory to establish immune status and, therefore, did not represent a cross-section of the population.

Rubella HI test. The HI test for the detection of rubella antibodies was performed according to the Centers for Disease Control (CDC) protocol by the trypsinized human O cell method and 4 U of antigen per 0.025 ml (10). Nonspecific inhibitors were removed with either heparin-MnCl₂ or dextran sulfate-CaCl₂. No heat inactivation is required in either standardized procedure. Heat inactivation, when performed, consisted of heating at 56°C for 30 min and was always done after the removal of lipoprotein inhibitors. The rubella HI antigen was obtained from Flow Laboratories, Rockville, Md. Dextran sulfate was obtained from Dextran Products Limited, Scarborough, Ontario, Canada. Reagent grade chemicals were used in all formulations. Tests were performed in disposable polystyrene plates with V-shaped wells (Linbro, Hamden, Conn.). Patterns of agglutination were read by roll (running buttons). Results were expressed as titers representing the reciprocal of the highest serum dilution totally inhibiting hemagglutination.

Sucrose gradient centrifugation. The method of Vesikari and Vaheri (13) standardized by the CDC (10) was used to separate serum immunoglobulins G and M. Undiluted, non-heat-inactivated, non-chemically treated sera (0.2 ml) were used for the gradients. The presence of immunoglobulins G and M in various fractions was determined by radial immunodiffusion using commercially prepared plates (Meloy, Springfield, Va.). The reconstitution experiments were done in the following way: non-heat-inactivated, non-chemically treated abnormally reacting sera (ARS) were fractionated by density gradient centrifugation. Each gradient fraction was divided into two aliquots; one was heat inactivated at 56°C for 30 min, and the other was not heated before testing for HI activity. Pooled
fractions 10 and 11 (heat-labile gradient fractions [HLGF]) were added to negative sera either before or after chemical treatment. The amounts used were as follows: 0.05 ml of serum or 0.4 ml of supernatant of chemically treated serum and 0.1 ml of HLGF either heat inactivated or not. Controls with phosphate-buffered saline instead of HLGF were included.

C depletion. Serum complement (C) activity was depleted in five ways. (i) Absorption with zymosan (Sigma Chemical Co., St. Louis, Mo.) was carried out by suspending 20 mg of zymosan in 1 ml of serum in the presence of 0.01 M Mg at and incubating the mixture at 37°C for 60 min with frequent shaking. The absorbant was removed by centrifugation. (ii) Removal of C activity achieved by incubating the serum at 37°C for 60 min with endotoxin, 2 mg of Escherichia coli O55:B5 lipopolysaccharide (Sigma) per ml. (iii) C depletion with cobra venom factor (Cordis Laboratories, Miami, Fla.) was achieved by using 100 U of cobra venom factor per ml of serum and incubating at 37°C for 30 min. Mg (0.01 M) was present in the reaction mixtures for both lipopolysaccharide and cobra venom factor treatments. (iv) C was also depleted with a crude preparation of protein A from Staphylococcus aureus (Sigma). This treatment involved incubation of the serum with 15 mg of protein A preparation per ml at 37°C for 60 min and removal of the absorbant by centrifugation. (v) Normal sera with no detectable HI antibody to rubella were used as the source of immunoglobulin G for C depletion. This human immunoglobulin G was prepared by ammonium sulfate precipitation at 50% saturation and further purification by diethylaminoethyl cellulose column chromatography. After promotion of aggregation by heating at 63°C for 60 min (5), this immunoglobulin G preparation was used for removal of C activity. The sera were incubated with aggregated immunoglobulin G at a concentration of 1 mg/ml at 37°C for 30 min.

C levels were determined in both treated and untreated sera; activity provided by the classical pathway was measured as described by Mayer (8), whereas alternative pathway activity was measured by the method of Plats-Mills and Ishizaka (11).

RESULTS

ARS. The interpretation of the rubella HI test using the CDC procedure was usually clear-cut. Negative sera showed no agglutination in the serum control, but agglutination was readily visible in all of the test wells. Positive sera also showed no agglutination in the serum control, but displayed inhibition of hemagglutination in one or more of the test wells (Table 1).

ARS yielded another pattern of results, different from the patterns described above. ARS showed the following typical characteristics: no agglutination in the serum control, agglutination in the test wells containing the lowest serum dilutions, lack of agglutination in the next one or two middle dilutions, and reoccurrence of agglutination in the higher dilutions that followed. ARS, as it will be demonstrated in this paper, represent a variation of false-positive reactions. The feature which distinguishes ARS from other false-positive reactions is the occurrence of agglutination in the lowest serum dilutions. This agglutination may occur at the 1:8 (ARS-8), 1:16 (ARS-16), or 1:32 (ARS-32) dilutions. Examples of different patterns of ARS are shown in Table 1.

Effects of physical treatment of ARS. Freezing and thawing of ARS often resulted in conversion to negative reactions. Some sera converted from ARS to negative after one freeze-thaw cycle, whereas other sera required additional cycles to eliminate abnormal reactions. ARS (220 sera) which were first treated for the removal of nonspecific inhibitors with dextran sulfate-CaCl2 and then heat inactivated were found to be negative in rubella HI tests (Table 2). When all sera giving positive reactions were heat inactivated it was determined that a relatively small proportion of what was presumed to be positive sera was also affected by heat. Heat inactivation of some of the sera titrating between 1:8 and 1:128 resulted in a loss of titer to <1:8. In contrast to the effect on false-positive sera and ARS, heat treatment did not affect rubella HI titers of truly positive sera (Table 2). Among 507 sera tested, 6.9% were ARS, 3.0% were false-positive, 0.9% were negative, and 89.2% were truly positive.

Demonstration of the negative nature of ARS and false-positive sera. Density gradient centrifugation was used to establish whether the dextran sulfate-CaCl2 treatment followed by heat inactivation was causing the removal of any rubella-specific antibody. This procedure resulted in the separation of nonspecific inhibitors from immunoglobulin-containing fractions (Fig. 1). Moreover, when four randomly selected ARS were tested for the presence of rubella neutralizing antibodies, they were found to be negative.

Relationship between divalent cation concentration and ARS. As demonstrated by the heat inactivation experiment (Table 2), ARS represent a variation of false-positive sera which contain a heat-labile inhibitor. The unique characteristic of ARS that distinguishes them from other false-positive sera is the agglutination occurring in the lower-dilution wells. The presence of this agglutination suggests that the heat-labile inhibitor is itself inhibited in wells containing higher serum concentrations. One conceivable explanation for this observation is that the agglutination in the lower-dilution wells of ARS is due to the concentration of divalent cations. We hypothesized that the remaining divalent cations impaired the action of the heat-labile inhib-
itor, thus resulting in viral agglutination. As the divalent cations were diluted out the heat-labile inhibitor was then able to manifest its action. We tested this hypothesis by first varying the concentration of calcium. The concentration of Ca$^{2+}$ in the dextran sulfate-CaCl$_2$ treatment was varied from 0.5 to 2.0 M with all other conditions remaining unchanged. Unheated and heat-inactivated samples of treated sera were titrated by HI. ARS which showed agglutination at 1:8 (ARS-8) when the standard 1.0 M Ca$^{2+}$ concentration was used in pretreatment became ARS-16 when the Ca$^{2+}$ concentration was increased to 2.0 M. Conversely, the same ARS-8 converted to being false-positive when the Ca$^{2+}$ concentration was reduced to 0.5 M. Titration after heat inactivation readily revealed the true negative nature of these sera. However, when truly positive sera were used, titers were found not to be dependent on Ca$^{2+}$ concentration. Examples of the effect of Ca$^{2+}$ concentration are shown in Table 3.

A widely used alternative method for the removal of nonspecific inhibitors from serum involves the combined use of heparin and MnCl$_2$. ARS and false-positive reactions were also common among sera treated with these reagents. It is of interest to note that the divalent cation Mn$^{2+}$ can also influence and distort the rubella HI test. The results (Table 4) indicate that abnormal patterns of agglutination were Mn$^{2+}$ dependent, and that some ARS and false-positive reactions were suppressed by higher Mn$^{2+}$ concentrations, whereas they were not suppressed by increased Ca$^{2+}$ concentrations. Again, heat inactivation revealed the true negative nature of both ARS and false-positive sera.

### Table 1. Agglutination patterns of negative sera, ARS, and positive human sera in titrations of rubella antibodies

<table>
<thead>
<tr>
<th>Reciprocal dilution of serum</th>
<th>Agglutination</th>
<th>Negative serum</th>
<th>ARS-8</th>
<th>ARS-16</th>
<th>ARS-32</th>
<th>Positive serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Serum control**

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### Table 2. Effect of heat inactivation on the titers of anti-rubella antibodies

<table>
<thead>
<tr>
<th>No. of sera</th>
<th>Titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative ARS</td>
<td>False-positive</td>
</tr>
<tr>
<td>120</td>
<td>&lt;8</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>19</td>
<td>64</td>
</tr>
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<td>1</td>
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<td>40</td>
<td>128</td>
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<tr>
<td>256</td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocal of the highest serum dilution showing HI.
° Sera were heated at 56°C for 30 min.
° ARS-8 indicates ARS, i.e., agglutination occurring at the 1:8 dilution, inhibition of agglutination at 1:16, and agglutination in the higher dilutions.
normal serum reactivity. Since it has been reported that certain viruses are inactivated by serum C (2, 9) and that the inhibitory phenomenon described here was both Ca\(^{2+}\) dependent and heat labile (conditions that also apply to the C activity via the classical pathway), the possible involvement of C in manifestation of abnormal serum reactivity was considered. To examine this possibility, a number of ARS were tested after treatment with various C inhibitors. Pretreatment of ARS with zymosan effectively removed the ARS condition, but inactivation of C by four other methods failed to do so (Table 5). Controls performed with positive sera, i.e., sera containing rubella antibodies, gave comparable titers before and after treatment with any of the C inhibitors used in our experiments.

Reconstitution experiments using gradient fractions from ARS and false-positive sera. ARS fractions separated by density gradient centrifugation were tested for reactivity in the HI test before and after heat inactivation of the fractions. Inhibition of agglutination was demonstrated only in the top fractions, i.e., those in which nonspecific inhibitors are found (Fig. 1). Inhibitory activity present in two of the lighter of the top fractions (10 and 11) was no longer detectable after heating at 56°C for 30 min. Inhibitory activity was only partially removed from fraction 12 after heat inactivation,
indicating the presence of both heat-labile and heat-stable inhibitor(s). Similar results were obtained when false-positive sera were fractionated.

Pooled fractions 10 and 11 (i.e., fractions demonstrating heat-labile activity only) from four gradients of ARS and false-positive sera (HLGF) were used for the reconstitution. When HLGF was added to negative sera, either before or after these sera were chemically treated, the ARS and false-positive phenomenon was reproduced with previously truly negative sera (Table 6).

**DISCUSSION**

These results indicate that the standard methods for removal of nonspecific serum inhibitors in the rubella HI tests do not adequately remove all inhibitors. We have demonstrated that in as many as 10% of the sera submitted to our laboratory for the determination of rubella antibody titers a heat-labile activity exists which impairs the correct reading of the tests, thus leading to the reporting of false-positive results in some instances.

Cremer reported on the occurrence of nonspecific agglutination in a rubella HI system using heparin-MnCl₂ and baby chicken erythrocytes (3) and pointed out the need to heat inactivate the serum for 15 min to circumvent this problem. This particular phenomenon reported by Cremer appeared to be one of nonspecific agglutination since it was observed in the serum control as well as in the test wells and only with chicken and not with trypsinized human erythrocytes. One plausible explanation given by the author was that the heparin-MnCl₂ mixture did not completely precipitate with the lipoprotein fractions of the serum; thus, the residual heparin-MnCl₂ remaining in the serum attached to the baby chicken erythrocytes used in the test, causing the cells to agglutinate. By heat inactivating the sera at 56°C for 15 min after the treatment with heparin-MnCl₂, an increased precipitation of the residual agent and serum inhibitors was observed. In our case, heating the sera at 56°C for 15 min was not sufficient to prevent the ARS and false-positive phenomenon; heating for 30 min was required. Although it seems clear that the phenomenon described in this paper and that reported by Cremer are different, we do not discard the possibility that aggregation and then precipitation of nonspecific inhibitors and the chemical used to eliminate them could be favored by heat treatment. In this regard the reconstitution experiments with the isolated heat-labile activity from the sucrose gradient (HLGF) favor the hypothesis of the presence of a heat-labile inhibitor(s) in certain sera that is nonremovable by chemical treatment. Moreover, it is also possible that both phenomena could take place at the same time in the same ARS.

It is possible that the problem with ARS may occur in many laboratories using either the dextran sulfate-CaCl₂ or the heparin-MnCl₂ method for treating sera in the rubella HI test. This problem has most likely been artificially minimized, however, by reducing the Ca²⁺ or Mn²⁺ concentration. This method of dealing with ARS has been suggested in the literature (1, 4). It is important to note that lowering the divalent cation concentration indeed reduces or eliminates the occurrence of ARS, but many of those sera are converted into false-positive sera with titers as high as 1:64 in some instances. Based on the results presented in this paper we do not recommend reducing the divalent cation concentration from the 1.0 M described in the CDC protocol (10).

As we demonstrated in this paper, when the CDC protocol is used (10) the incidence of false-positive sera amounts to 3%. If the divalent cation concentration is reduced, the ARS are converted into false-positive sera, and the combined incidence would increase the appearance of false-positive sera to as high as 10%. This problem of false rubella HI titers using the dextran sulfate-CaCl₂ treatment procedure was also recently pointed out in a report of the Wisconsin State Laboratory of Hygiene to the participating laboratories in their rubella evaluation program. A feasible explanation for such a high incidence could be based on the appearance of false-positive titers when heat inactivation is not included in the test.

We also would like to suggest that the appre-
ciable number of false-positive sera could explain some reports (6, 7) questioning the significance of some low rubella HI titers. One such report documented that three women with low titers of rubella HI antibodies went on to contract rubella (7). Another instance has been reported wherein three patients with detectable HI antibodies to rubella (two with titers up to 1:160) were shown to have negative rubella HI titers at a later date (6). The latter situation was also observed in our laboratory before the time when heat inactivation of the treated serum dilution was instituted as a standard procedure.

Although the exact nature of the heat-labile inhibitor(s) was not determined by this investigation, our results indicate that the inhibitory activity was not dependent on C. The abnormal serum reactivity persisted after depletion or inhibition of C by various independent means, including the treatment of sera with endotoxin, protein A, cobra venom factor, or immunoglobulin aggregates. Zymosan did remove the reactivity, probably due to its capacity to remove lipids rather than its anti-C properties. The heat-labile inhibitor(s) was found to be a light-density component of serum as demonstrated with the density gradient experiments.

It would seem that the presence or absence of the heat-labile inhibitor herein described could be dependent upon the conditions surrounding serum collection or on the physiological state of the individual being tested. We do not yet know, for example, whether there is a lower incidence of ARS when the sera are collected from individuals in the fasting state.

Further work is needed to determine the nature of the heat-labile inhibitor(s) and the condition under which it is present in serum. Based on our findings, however, we emphasize that heat inactivation must follow chemical treatment of sera and should be incorporated into the standard rubella HI test procedure. Doing so will eliminate both the ARS reaction and related false-positive reactions.

LITERATURE CITED