Rubella Hemagglutination-Inhibition Test: False-Positive Reactions in Sera Contaminated with Bacteria

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We have shown that bacterial contamination of sera can have a marked influence on the results of the rubella hemagglutination-inhibition (HI) test. In addition to increasing the levels of nonspecific agglutinins, a number of common bacterial species tested had a significant effect on the HI titers. When sera free from rubella-specific antibodies were contaminated with Bacillus subtilis or Pseudomonas fluorescens, HI titers ranged from 16 to 128 (expressed as the reciprocal of the highest serum dilution completely inhibiting hemagglutination) after treatment with heparin/MnCl₂. Our observations demonstrate, therefore, that bacterial contamination can be one of the causes of false-positive reactions in this test.

It is a widespread practice in diagnostic laboratories to collect blood samples into nonsterile tubes (such as vacuum blood-collecting tubes) and to store the separated sera or plasmas in clean, but nonsterile, containers. It is not surprising, therefore, that a certain proportion of these samples become contaminated with bacteria. Yet little attention has been paid so far to the potential effects of these bacterial contaminants and their products on the constituents of serum or plasma.

In a study of the lipid patterns of contaminated sera, it was shown that in certain samples there was a decrease in lipoproteins and phospholipids and a concurrent increase in serum triglycerides. Phospholipase C-producing, gram-positive, aerobic, sporeforming bacilli were isolated from these samples (13). Treatment of serum with phospholipase C has been reported to be an effective way of inactivating the lipoproteinaceous nonspecific inhibitors (NSI) of rubella virus hemagglutination (HA) (5, 8). It might appear, therefore, that for the rubella hemagglutination-inhibition (HI) test, bacterial contamination of this sort would be advantageous rather than the opposite. However, partial hydrolysis of the phospholipid moieties by this enzyme has been found to interfere with the formation of insoluble complexes between β-lipoproteins and heparin/metal cation mixtures (15), and treatment of serum with heparin/MnCl₂ is one of the most widely used methods for the removal of lipoprotein NSI of rubella HA. It is possible, therefore, that contamination with phospholipase C-producing bacteria may result in the incomplete removal of NSI, thus producing false-positive reactions.

Two situations in which bacterial contamination produced false positives in the rubella HI test have, in fact, been reported. White and Tinnion (17) observed that sera contaminated by an organism closely resembling Pseudomonas fluorescens contained a nonspecific inhibitor of rubella HA that was not removed by treatment with heparin/MnCl₂. Our observations demonstrate, therefore, that bacterial contamination can be one of the causes of false-positive reactions in this test.

In addition to affecting the levels of NSI, there is also the possibility that certain bacterial contaminants can reduce the concentration of specific immunoglobulins. For example, most strains of Staphylococcus aureus contain a cell wall protein, the so-called protein A, which complexes with the Fc portion of immunoglobulin G (IgG) molecules. Sufficiently large quantities of S. aureus might cause an appreciable reduction in rubella-specific IgG levels, and this method has, in fact, been used for the removal of IgG in order to permit direct determination of specific IgM levels (3).

Yet another way that bacterial contamination might affect the rubella HI test is by the
production of nonspecific agglutinins (NSA). For example, three strains of enteric bacteria were found to agglutinate the blood cells of a number of animal species (16). Since human sera do not normally contain agglutinins for trypsin-treated human cells, use of these cells as indicators in the rubella HI test obviates the need for prior absorption of the sera with packed erythrocytes (11, 12). Introduction of NSA by bacterial contamination, therefore, might mask low specific antibody titers and would necessitate repeating the test after absorption of the agglutinating material.

To investigate these possibilities more closely, we initiated a study of the effect of common bacterial contaminants on the rubella HI test. The preliminary results reported here indicate that whereas some common bacterial species have no deleterious effects, others can influence the assay results to a marked degree and may be responsible for some of the reports of false positives that limit the usefulness of the assay method.

MATERIALS AND METHODS

Rubella HA and HI tests. Rubella HA and the HI tests were carried out as in previous work (6), using the modified Center for Disease Control (CDC) protocols with trypsin-treated human O cells as indicators (6, 10, 11). Nonspecific inhibitors were removed by treatment with heparin/MnCl₂ as directed in the CDC procedure except that 0.75 M MnCl₂ was used instead of the recommended 1.0 M solution. This decreased the incidence of nonspecific agglutination. The sera were not absorbed with blood cells before assay.

Human type O, Rh-negative blood was obtained from a single donor (J.B.C.) and was stored in Alsever solution at 4°C for up to 3 weeks before use. Trypsinization of the erythrocytes was carried out by the method of Quirin et al. (11), using 2% crystallized trypsin (Sigma Chemical Co., St. Louis, Mo.). The trypsinized cells were stored in dextrose-gelatin-veronal (DGV) buffer, pH 7.3, for a maximum of 7 days before use.

Rubella HA antigen, lot number 2020-1, was purchased from Connaught Diagnostics, Willowdale, Ontario. It had been prepared from the HPV-77 strain of virus by Tween-ether extraction, and was obtained as a lyophilized preparation with a titer of 1.38 HA units. Sodium heparin (5,000 U/ml) and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate)-buffered diluent (HEPES-saline-albumin-gelatin), pH 6.2, were also obtained from Connaught Diagnostics. Alsever solution and dextrose-gelatin-veronal buffer were made up as recommended in the CDC procedure (10). Reagent-grade chemicals were used in all formulations.

Assays were carried out in rigid polystyrene V plates (Linbro Chemical Co., New Haven, Conn.), using Microtiter droppers and diluters (Cooke Engineering Co., Alexandria, Va.). All titers were expressed as the reciprocal of the highest serum dilution that totally inhibited hemagglutination.

Bacteria. Pure cultures of bacteria were obtained from the Department of Microbiology and Parasitology teaching collections. Sera were tested for bacterial contaminants by streaking loopfuls on blood agar plates and incubating these at 25°C for 48 h. A general identification of isolated colonies was then made by Gram staining, by colony morphology, and by the presence or absence of hemolysis on the blood agar plate. Further classification was made in some cases by biochemical tests.

Sera to be contaminated with a particular bacterium were inoculated with loopfuls of plate cultures (1 loopful/0.6 ml of serum). The samples were then left at 25°C for 48 h before they were assayed for HI activity.

Sera. Most of the sera used in the present study were clinical specimens drawn in several diagnostic laboratories for serum chemistry or rubella HI antibody assays. All sera were from blood collected by venipuncture into nonsterile Venoject vacuum blood collecting tubes, without additives (Jintan Terumo Co., Ltd., Tokyo, Japan), which were allowed to clot at room temperature for 6 to 12 h before separation. Further details of the storage conditions of the sera are described in Results.

RESULTS

Bacterial contamination of sera. On three separate occasions, batches of clinical serum specimens were tested for bacterial contamination. All sera were collected as described in Materials and Methods.

The first batch of 98 sera had been stored in clean but nonsterile, unused glass test tubes taken straight from the manufacturer's carton. The samples were left uncovered at room temperature for up to 6 h and then stored at 4°C for about a week in racks covered with Parafilm. When plated on blood agar, 3 of the 98 sera (3.1%) were found to contain bacteria. These appeared to be a mixture of coliforms, diphtheroids, and staphylococci, with the gram-negative rods predominating.

In another survey, of 95 sera collected in reused, detergent-washed Venoject tubes and stored at 4°C for 1 to 3 days, 15 (16%) showed significant levels of contamination. The predominant organism among these (8/15) was a diphtheroid (Corynebacterium species), with the remainder of the samples being contaminated with Alcaligenes faecalis (4/15), a coliform (1/15), and a staphylococcus (2/15).

Over a period of several months, all sera assayed for rubella-specific HI antibodies that showed the presence of nonspecific agglutinins were stored at −20°C for further testing. Most of these samples had been stored in reused, detergent-washed Venoject tubes. When tested on blood agar plates, 24 out of 68 (35%) were
found to be contaminated with bacteria, some samples with more than one species. Coliforms and other enterobacteriaceae predominated, although staphylococci were also quite prevalent.

Effect of various bacteria on the rubella HI test. From the work described in the introduction, the possibilities existed that contamination of serum with certain bacteria might result in: (i) the production of NSA; (ii) a decrease in the specific antibody titers; and/or (iii) an increase or decrease in the level of NSA.

To eliminate the possible involvement of naturally occurring agglutinins, serum was collected aseptically from the donor who provided the erythrocytes for the HI test. Samples of this serum were inoculated with the various bacteria and incubated for 2 days at 25°C before being assayed for rubella-specific HI antibodies and total HI (i.e., rubella-specific HI plus NSI). Serial twofold dilutions of the serum controls were also prepared so as to provide a measure of the levels of NSA. Results are summarized in Table 1. It can be seen that many of the bacteria tested produced NSA that were not removed by treatment with heparin/MnCl₂.

The uncontaminated serum was shown on several occasions to have a specific rubella HI titer of 256. Two bacteria (Escherichia coli and Streptococcus faecalis) reduced this titer significantly (a fourfold increase or decrease being considered significant). One bacterium, B. subtilis, increased the apparent antibody titer significantly. That this apparent increase in specific antibody titer was actually due to an increase in the activity of the NSI was shown in the following experiment.

Three organisms were chosen for further study: P. fluorescens, B. subtilis, and S. aureus. Serum samples from eight different patients, shown previously to be free of rubella-specific antibody, were inoculated with one or the other of these organisms. After 2 days at 25°C, they were assayed for rubella-specific and total HI as described above. Results are shown in Table 2.

The uncontaminated controls showed a low level of NSA in one of the eight samples, and total HI titers ranged from 128 to 4,096. These are normal ranges in our system. Treatment of the sera with heparin/MnCl₂ removed all traces of NSA and NSI. Contamination with S. aureus appeared to reduce the NSI levels, and the remainder was removed completely after treatment with heparin/MnCl₂. With the other bacteria, however, the situation was quite different. P. fluorescens produced a low level of NSA in all the sera and increased the NSI in most of them. After treatment with heparin/MnCl₂, all except one serum showed residual NSI. This was even more evident in the sera contaminated with B. subtilis. This bacterium produced very high levels of NSA, and the end points of the NSI were not reached at serum dilutions of 1:16,384. As with the P. fluorescens-contaminated samples, heparin/MnCl₂ was unable to remove all nonspecific inhibitory activity.

DISCUSSION

One of the drawbacks of the rubella HI test is that there is no completely satisfactory way of ensuring that what is being measured is actually (and solely) specific antibody. By the incorporation of suitable controls in the assay, the presence or absence of NSA can be readily monitored. We routinely test our serum controls through a dilution of 1:32 to permit some degree of quantitation of the level of NSA. It is a much more difficult task to monitor the efficiency of the methods for removal of NSI. Un-

### Table 1. Effect of different bacteria on the rubella HI test

<table>
<thead>
<tr>
<th>Bacterial contaminant</th>
<th>Titers before treatment with heparin/MnCl₂</th>
<th>Titers after treatment with heparin/MnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSA</td>
<td>HI</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>-</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
<td>8</td>
<td>2,048</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>&gt;32</td>
<td>≥2,048</td>
</tr>
<tr>
<td>B. cereus</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Klebsiella aerogenes</td>
<td>8</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Salmonella gallinarum</td>
<td>-</td>
<td>≥2,048</td>
</tr>
<tr>
<td>Control (sterile)</td>
<td>-</td>
<td>2,048</td>
</tr>
</tbody>
</table>

* = Negative (<8).
less physical techniques such as density gradient or flotation ultracentrifugation are used, there is no way of determining whether all the NSI of rubella HA have been separated from specific antibody. Even these methods are not infallible, since NSI have been found in fractions containing IgG (7) and IgM (1) after sucrose gradient ultracentrifugation, and flotation methods are based upon the assumption that all inhibitors have the properties of lipoproteins. In addition, these methods would not be suitable for routine diagnostic use.

Within the confines of the HI test itself, the most satisfactory method of monitoring the removal of NSI is to include among the controls a negative serum that contains very high levels of NSI. If this serum repeatedly registers as negative in the test assay, it is likely that equal or lesser amounts of NSI present in other sera will also be effectively removed. This is probably a reasonable assumption in the case of sterile sera. Our data clearly demonstrate, however, that if the sera are contaminated with bacteria, false-positive reactions may be produced.

At present, we do not know whether the bacteria modify and enhance existing inhibitors or whether they produce different ones. For the purposes of routine diagnosis of rubella infection by the HI test, however, the problem is not a pressing one. It can even be avoided completely by ensuring the sterility of the sera before testing. In situations where total sterility is impracticable, the chances of bacterial contamination can be reduced by allowing the blood to clot at 4°C and maintaining the sera at this temperature until assayed. Allowing the blood to clot at this temperature has the added advantage that cold agglutinins, if present, would probably be removed by adsorption onto the erythrocytes (14).

Our data indicate that contamination of sera with several different bacteria can result in the production of low levels of nonspecific agglutination (high levels in the case of B. subtilis) that may not be removed by treatment with heparin/MnCl2. The extent of NSA production, of course, would depend on a number of variables affecting bacterial growth, including the storage conditions of the sera and the presence or absence of antibiotics. Not all instances of NSA occurrence are due to bacterial contamination, however; other factors almost certainly contribute. For example, Biano et al. (4) have shown that an excess of manganous chloride can agglutinate erythrocytes. If a serum sample has only low levels of heparin/MnCl2-precipitable material, sufficient manganous ions may

### Table 2. Effect of bacterial contamination on rubella HI test results

<table>
<thead>
<tr>
<th>Serum sample number</th>
<th>S. aureus</th>
<th>B. subtilis</th>
<th>P. fluorescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (sterile)</td>
<td>8</td>
<td>1.024</td>
<td>8</td>
</tr>
<tr>
<td>Untreated</td>
<td>128</td>
<td>2.048</td>
<td>&gt;2,048</td>
</tr>
<tr>
<td>Hep/MnCl2</td>
<td>16384</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>NSA</td>
<td>1024</td>
<td>1024</td>
<td>32</td>
</tr>
<tr>
<td>NSA</td>
<td>16384</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>NSA</td>
<td>1024</td>
<td>1024</td>
<td>32</td>
</tr>
<tr>
<td>NSA</td>
<td>16384</td>
<td>256</td>
<td>512</td>
</tr>
</tbody>
</table>

- **NSA**: Negative (<8)
- **Ther. obs. by NSA**: Thermo. resistant by NSA.
remain in the treated supernatant to produce this effect. We have found that reduction of the MnCl₂ concentration from the CDC-recommended 1.0 to 0.75 M decreases the incidence of NSA without having any deleterious effect on the removal of NSI. The NSA produced by the bacteria we have studied can be removed by absorption of the sera before assay with packed erythrocytes. However, one of the advantages of using trypsin-treated human erythrocytes as indicator cells is that routine serum absorption is unnecessary. Reintroduction of this step due to bacterial contamination, therefore, eliminates this advantage.

Since bacterial contamination undoubtedly increases the incidence of nonspecific agglutination, we feel that use of the human cell system provides a diagnostic advantage over systems requiring routine serum absorption (such as those using day-old chick cells). In those cases where levels of NSA are produced by bacterial action, routine absorption may actually contribute to the problem of false positives, since the NSA will be removed but the increased levels of NSI may not be, and may therefore go undetected. Our observations indicate that if nonsterile sera are being tested, any level of NSA warrants caution in the interpretation of results. Certainly, the cause of high levels of agglutination should be investigated further.

LITERATURE CITED