Rubella Hemagglutination Inhibition: Removal of Nonspecific Agglutination Due to Manganese Chloride

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Nonspecific inhibitors of rubella hemagglutination can be removed by treatment of sera with heparin-manganese chloride for use in the hemagglutination-inhibition test. After removal of nonspecific inhibitors by this procedure, an excess of manganese chloride may remain. This may cause the cells to agglutinate, thus obscuring the reading at low serum dilutions. This disadvantage can be overcome by the addition of sodium carbonate, which forms an insoluble compound with manganese chloride and does not interfere with antibody determination. The procedure presents a further refinement of the hemagglutination inhibition test for rubella by increasing specificity and sensitivity; it permits detection of antibody levels as low as 1:4 in sera.

The most significant sources of error associated with the hemagglutination inhibition (HI) test for antibody to rubella virus arise from the presence of non-immunoglobulin inhibitors of rubella hemagglutinin (they are present in almost all sera at titers that range from 1:4 to 1:200) and from the procedures used to remove them (4). Inhibitors have been removed by a number of methods. One of these involves the use of heparin-manganese chloride (H-MnCl₂; [5]). Although precipitation of inhibitors with H-MnCl₂ is specific in its action and generally reliable when carried out by skilled personnel, it is often complicated by the occurrence of nonspecific hemagglutination, which obscures the reading of the test at low serum dilutions despite repeated absorption of the serum with 50% chick erythrocytes. This disadvantage may lead to serious error when the immunity status of pregnant women is considered, by preventing detection of antibody at low serum dilutions.

Attempts have been made to correct this drawback (3). By reducing the concentration of MnCl₂, nonspecific agglutination was minimized, but the nonspecific inhibitors were not always completely removed. The heating of the serum at 56°C for 15 min prior to absorption with chick erythrocytes also reduced the appearance of nonspecific agglutination but did not eliminate it.

This study is concerned with determining the cause of nonspecific agglutination in the H-MnCl₂ procedure and with attempting to correct it without interfering with antibody determination. The details of the procedure and a comparative study of its application in the HI test are described. (This material was presented in part at the 73rd Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 1973.)

MATERIALS AND METHODS

The HI test recommended by the Center for Disease Control was followed (6). For removal of inhibitors from serum we used H-MnCl₂, and then natural agglutinins were removed from sera by absorption with 50% chick erythrocytes. Rubella antigen was obtained commercially from the Grand Island Biological Company (Grand Island, N.Y.; rubella hemagglutination antigen lyophilized, lot numbers 028 and 035). HEPES-bovine serum-albumin-gelatin buffer (HSAG) was used throughout as diluent (Flow Laboratories, Rockville, Md.). Erythrocytes from unfed chicks. (Av-An Inc., Allston, Mass.) were washed in dextrose-gelatin-veronal buffer, and a suspension of 0.25% was made in the diluent buffer HSAG.

The microtiter system was used; disposable V plates, and reagents were kept cold. Mixtures of serum and 4 U of antigen were incubated at 4°C for 1 h before addition of erythrocytes. The completed tests were read after incubation at 4°C for 2 h or overnight.

Controls run with each test included sera containing a high titer of antibody, a low titer of antibody, and no detectable antibody, but containing a high titer of inhibitors. The reciprocal of the highest dilution of serum that showed complete inhibition of rubella hemagglutination was taken as the titer.

Indirect fluorescent tests for antibody were performed by the technique of Brown et al. (1), except
that a continuous line of green monkey kidney cells (Vero) was infected as a source of antigen (virus from a BHK-21 carrier line, courtesy of Monroe Vincent of Microbiological Associates, Bethesda, Md.). Rubella virus-infected Vero cells grown on coverslips were overlaid with 1:4 and 1:8 dilutions of test serum. This was followed by staining with fluorescein-conjugated anti-human globulin.

Sera were collected for a prospective study on the occurrence of reinfection by rubella virus in pregnant women with low titers of antibody (S. A. Biao, manuscript in preparation). We selected women whose hemagglutination inhibition titer was 1:16 or less at the first prenatal visit. A second specimen was drawn at delivery. Cord blood was also obtained from the newborn. Paired sera from women and cord were run in the same test.

RESULTS AND DISCUSSION

When equal volumes of chick erythrocytes were mixed with serial dilutions of various agents, heparin alone, or a mixture of heparin with serum, did not produce any agglutination (Table 1). The nonspecific agglutination was present when 1 M manganous chloride alone or with serum was mixed with chick erythrocytes, causing agglutination in dilutions up to 1:8 to 1:16. This suggests that after heparin removal the nonspecific inhibitors (β-lipoproteins) in the presence of divalent cations such as MnCl₂ (6) an excess of MnCl₂ may remain in the diluted serum, causing the agglutination of chick erythrocytes. By reducing the concentration of MnCl₂ to less than 1 M, the nonspecific inhibitors may not always be removed; consequently, we tried to concentrate our attention on finding an agent that binds the excess MnCl₂ without causing a change in specific antibody titer. By adding sodium carbonate (Na₂CO₃), the nonspecific agglutination was eliminated (Table 1). The excess of MnCl₂ was precipitated by Na₂CO₃, and the resulting insoluble compound, MnCO₃, was removed by centrifugation. Best results were obtained by using a horizontal centrifuge at 900 × g. The new procedure for preparing the sera for the hemagglutination inhibition test is as follows: Mix 0.2 ml of serum with 0.3 ml of diluent buffer (described in Materials and Methods). Add 0.2 ml of the solution of 5,000 U of heparin and 1 M MnCl₂ (1:1) and mix. Incubate at 4°C for 15 min and shake at 5-min intervals. Add 0.2 ml of 50% chicken erythrocytes and mix. Incubate at 4°C for 1 h, and shake gently at 15-min intervals. Centrifuge at 900 × g for 15 min at 4°C and pipette the supernatant. Add 0.05 ml of 1 M Na₂CO₃ and shake well for 1 min. Centrifuge at 900 × g for 25 min at 4°C, and pipette the supernatant. The final dilution is approximately 1:4.

The treated sera obtained were clear, or slightly turbid due to a precipitate which did not interfere with the HI test. Treated sera could be stored at 4°C for up to 2 weeks before testing if evaporation was prevented.

To find out whether the addition of Na₂CO₃ or the resulting MnCO₃ had an effect on antibody determination, we used this procedure to study a group of pregnant women selected from a long-term prospective study on rubella reinfection occurring during pregnancy. These women had already been screened at their first prenatal visit for immunity to rubella virus by standard HI procedure. A number of them had shown nonspecific agglutination at the 1:8 serum dilution; their titers were recorded as less than 1:16 and they were considered as nonimmune.

Of 2,000 sera tested by the standard procedure, 573 showed a titer of less than 1:16, which means that 28.6% had no demonstrable antibody. The 573 women were followed, and a second specimen was drawn at delivery time, as well as cord blood from the infant. Paired maternal sera and cord were run in the same manner.

**Table 1. Nonspecific hemagglutination by MnCl₂**

| Treatment                          | Hemagglutination* |
|-----------------------------------|-------------------|
|                                   | 1:2               | 1:4   | 1:8   | 1:16  | 1:32  |
| Heparin (5,000 U/ml)              | 0                 | 0     | 0     | 0     | 0     |
| Heparin + serum (equal volume)    | 0                 | 0     | 0     | 0     | 0     |
| MnCl₂ (1 M)                       | 4+                | 4+    | 4+    | +     | 0     |
| MnCl₂ (1 M) + serum (equal volume)| 4+                | 4+    | 2+    | 0     | 0     |
| H-MnCl₂ + serum (equal volume)*   | 4+                | 4+    | +     | 0     | 0     |
| H-MnCl₂ + serum + Na₂CO₃*         | 0                 | 0     | 0     | 0     | 0     |

* Equal volume of 0.25 newborn-chick cells and dilutions of various agents in dextrose-gelatin-veronal buffer.
* Hemagglutination, 0 to 4+.
* After removal of precipitate by centrifugation.
test by the procedure described above. Of the 28.6% of the women initially showing an HI titer of less than 1:16, more than half had a titer of 1:8 or 1:4, and less than half showed a titer of less than 1:4 (Table 2). This means that more than half of the women originally considered “nonimmune” proved to have low titers of antibody, and the group of women susceptible to rubella was smaller. The results on 300 cord sera corresponded with maternal sera.

Despite the use of the H-MnCl₂-Na₂CO₃ procedure and repeated absorption with chick erythrocytes, for some unknown reason we failed to eliminate nonspecific agglutination in the 1:4 dilution of 2 to 4% of the sera and cord bloods, and therefore we were not able to read the 1:4 dilution.

To investigate the reliability of the test and to answer the question of whether low HI titers were truly antibody, a comparison was made with immuno-fluorescent antibody determination. Sixteen sera collected from women at delivery and 16 cord sera from their infants were coded and tested by immuno-fluorescence. There was agreement in titers of antibody (Table 3). In only one set out of 16 (specimen 409) the results were different; they were positive on fluorescent antibody and negative on HI in repeated tests, but the sera were found to have bacterial contamination. Thus, specificity of low levels of HI antibody was confirmed by indirect fluorescence, and these data suggested a high correlation between the two tests.

With a more sensitive HI test, we may be able to better document reinfection in individuals with low antibody levels, and also perhaps to determine that antibody level which protects the fetus.

| Specimen no. | Adult sera | Cord sera |
|--------------|------------|-----------|
|              | HI titer   | Fluorescent antibody | HI titer |
|              | 1:4 | 1:8 | 1:4 | 1:8 |
| 119          | <4  | -   | <4  | -   |
| 210          | <4  | -   | <4  | ±   |
| 244          | 4   | +   | 4   | +   |
| 281          | <4  | +   | <4  | ±   |
| 409          | <4  | -   | <4  | -   |
| 419          | <4  | -   | <4  | -   |
| 429          | <4  | -   | <4  | -   |
| 503          | 8   | ND  | 16  | ND  |
| 525          | 8   | ND  | 8   | ND  |
| 590          | 8   | ND  | 8   | ND  |
| 1043         | <4  | -   | <4  | -   |
| 1044         | 8   | ND  | 8   | ND  |
| 1437         | <4  | -   | <4  | -   |
| 1998         | <4  | -   | <4  | -   |
| 2170         | 4   | +   | 4   | +   |
| 2226         | 4   | +   | 4   | +   |

* - Negative; ±, equivocal; +, positive; ND, not determined.

The modified H-MnCl₂-Na₂CO₃ procedure for removing nonspecific inhibitors was easy to perform and effective. It combined sensitivity with specificity in HI antibody determination.

**LITERATURE CITED**

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