DETECTION OF RUBELLA BY HEMAGGLUTINATION-INHIBITION


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U.S. Cl. 424—12

9 Claims

ABSTRACT OF THE DISCLOSURE

Method of detecting the presence of rubella antibodies in blood by hemagglutination-inhibition. Serodiagnosis of rubella is accomplished by mixing a sample to be tested, which can be blood serum or gamma globulin, with a rubella strain of virus in a red blood cell suspension from chicken, preferably chick less than 24 hours old, goose, or sheep and observing the same to determine if hemagglutination is inhibited.

This invention is concerned with a method of serodiagnosis of rubella. More specifically, this invention is concerned with a method of detecting the presence of rubella antibodies in blood by hemagglutination-inhibition.

The rubella virus infection, commonly known as German Measles, is primary a disease of children and young adults and is clinically characterized by sore throat, coryza, headache, malaise, myalgia, posterior cervical lymphadenitis, and a pale, pink macular rash. Rubella infection is highly contagious. The virus is probably communicated via the respiratory route by close personal contact and is usually detectable both in the blood and in nasopharyngeal washings.

The usual course of the disease leads to prompt and complete recovery although relapse occurs in 5–8 percent of the cases. Secondary bacterial infections are rare as are other complications which include arthritis, neuritis, gingivitis, thrombocytopenic purpura, and heart block. Meningoencephalitis occurs in one of every 6,000 cases and is 20 percent fatal. A patient who has contracted, and has recovered from, a rubella infection usually enjoys lasting immunity from subsequent attacks.

Accurate information on the incidence of the disease is not available because study has been limited by the virus' restrictive host range (humans and monkeys) and by the difficulty in diagnosing so mild a disease. Rubella is difficult to diagnose because of other unrelated rash diseases and because many cases of German Measles fail to develop recognizable signs of infection.

In pregnant women, precise diagnosis of rubella is essential since the most dismal aspect of the disease is that it is the only viral infection known to be associated with fetal abnormalities in cases where a pregnant woman contracts the disease in her first trimester of pregnancy. Though it is not true, by any means, that abnormalities always result in the offspring of a first-trimester infected pregnant woman (she has a 90 percent chance of bearing a normal baby), when abnormalities do occur, they are frequently so severe as to lead to intruterine fetal death, stillbirth, or delivery of a viable infant with tragic defects such as microencephaly, dental hypoplasia, blindness due to cataract formation, deafness due possibly to agenesis of the organ of Corti and acyanotic cardiovascular disease such as patent ductus arteriosus and intraventricular septal defects.

Considerable research has been done over a period of years toward developing a simple, reliable test for detecting immunity to rubella. The various tests which have been developed, such as complement fixation, neutralizing antibody, and fluorescent antibody methods, have been only partially successful and have had many drawbacks. The neutralization test, while providing the most reliable and useful information is expensive, complicated and time-consuming. Also, neutralizing antibody tends to be low in rubella, leading to difficulties in interpretation of results. The fluorescent antibody method is beset by similar problems. For these reasons, neither test is ideally suited to large scale use in diagnostic laboratories. While the complement fixation technique is relatively simple and provides results in 24 hours, the test is less sensitive than the neutralizing antibody test since rubella patients frequently fail to develop detectable CF antibody. Moreover, even when CF antibody appears, it is relatively transient. Thus, the method does not lend itself to epidemiological studies or to determining immunity in adults. Since attenuated rubella virus infections rarely evoke a CF antibody response, the test has no value in live vaccine studies.

Accordingly, it is a basic object of this invention to provide a method for the serodiagnosis of rubella free of the aforementioned and other such disadvantages.

Thus, it is a primary object of this invention to provide a technique for detecting the presence of rubella antibodies in blood which is technically simple, quick, highly sensitive and reliable. Additionally, it is an important object of this invention to provide a method for serodiagnosis of rubella which requires only inexpensive reagents whereby it can be readily used extensively.

The rubella virus antibody test of this invention can generally be used for: (1) diagnosis of infection, (2) detection of immunity (often long after infection), and (3) evaluation of vaccine efficacy.

Recently, a technique has been developed for attenuating rubella virus to produce a rubella vaccine capable of immunizing against the disease without inducing side effects. Reference may be made to copending application Ser. No. 603,239, filed Dec. 19, 1966 assigned to the same assigned as the instant application, the disclosure of which is incorporated herein in its entirety, for a more detailed description of this development. The availability of a rubella antibody detection test such as the instant invention provides is of significance in testing the usefulness of such vaccines and in speeding further development of the same.

Additionally, the techniques hereof are of particular importance in testing the immunity of pregnant women to rubella in view of the dangers to such individuals described hereinabove. In fact, this procedure can be utilized as a standard premarrige test so that women who are not immune to rubella can seek vaccine or other treatment prior to pregnancy to preclude the possibility of rubella infection.

Other and further objects hereof will be obvious or will become apparent from a consideration of the following detailed description.

Basically, the method hereof comprises mixing a sample to be tested, which could be the blood serum from the

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patient or gamma globulin, with a rubella virus antigen and adding erythrocytes from one of the following: chick less than 24 hours old), adult chicken, goose, and sheep, and visually determining whether or not hemagglutination has occurred.

Hemagglutination, or red blood cell clumping, has been used successfully in studies on influenza and other diseases. This phenomenon has not, however, been demonstrated to a significant extent in viruses or rubella. We have found that special preparations of rubella virus will cause the red blood cells of the above sources, and particularly of newly hatched chicks, to clump. When a sample of blood drawn from a person immune to German Measles is added to the clumped red blood cells, the red cell agglutination is inhibited. The rubella antibodies may be present in the blood because of exposure to German Measles or the use of a rubella vaccine.

**PRODUCTION OF HEMAGGLUTINATION ANTIGEN**

The M33 and ML strains of rubella virus were employed in production of hemagglutination (HA) antigens. The tissue cultures used for preparing the HA antigens were the WT-1 line of baby hamster kidney cells (BHK). The BHK cells were grown in monolayer cultures at 35°C in Eagle’s medium containing 10 percent fetal bovine serum, glutamine (200mM), and antibiotics.

The antigens were prepared in monolayer cultures of BHK cells grown in 32 oz. bottles. The medium was removed from the culture vessels and 5–10 ml of inoucullum containing 10^6 to 10^7 tissue culture infectious doses (TCID50) of BHK cell-adapted virus was added. After a 4-hour adsorption period at 35°C, each culture received 40 ml of maintenance medium consisting of Eagle’s medium containing 2 percent heat inactivated fetal bovine serum, which had been treated with kaolin by the method of Clarke et al., Am. J. Trop. Med. Hyg., 7, 1958, pp. 561–573, in order to remove non-specific inhibitors of hemagglutination. The maintenance medium was harvested from the cultures at daily intervals and tested for HA activity. The antigen-containing fluids were maintained at 4°C during processing and stored at −70°C. Control materials were prepared similarly from unoinoculated tissue cultures.

**PREPARATION OF ERYTHROCYTE SUSPENSIONS**

The following kinds of red blood cells (RBC) were examined in the HA test: chick (less than 24 hours old), adult, day-old, black, sheep, human type O, rhesus monkey, and guinea pig. For daily use, RBC suspensions were washed 3 times and resuspended in dextrose-gelatin-veronal buffer (DGV) as made by Clarke et al., supra, containing 0.2 percent bovine plasma albumin (BPA) at pH 7.3. Chick cells were used in 0.16 percent suspension; all other cells tested were resuspended in final concentrations of 0.5 percent.

**EXAMPLE I**

Tests for hemagglutination were performed in microtiter U disposable plastic plates by the method described by Sever in J. Immunol., 88, 1962, pp. 320–329. Serial two-fold dilutions of antigen were made in 0.025 ml volumes of the pH 7.3 DGV–BPA. The BPA suspensions were added in equal volume and the plates were incubated at 4°C and read after a 1 to 1.5 hour settling period. One unit of antigen was considered as the highest dilution which produced a pattern of complete hemagglutination. The procedure was repeated at room temperature (25°C) and 37°C. The results are shown in Table I.

**TABLE I—EFFECT OF DONOR SPECIES AND INCUBATION TEMPERATURE ON HEMAGGLUTINATION BY RUBELLA VIRUS**

<table>
<thead>
<tr>
<th>Type of red blood cell</th>
<th>37°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult chicken</td>
<td>1-2</td>
<td>3-4</td>
</tr>
<tr>
<td>Sheep</td>
<td>5-6</td>
<td>7-8</td>
</tr>
<tr>
<td>Human type O</td>
<td>9-10</td>
<td>11-12</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>13-14</td>
<td>15-16</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>17-18</td>
<td>19-20</td>
</tr>
</tbody>
</table>

1 Five lots of pooled chick red blood cells.
2 Titers of highest antigens dilution showing complete hemagglutination.
3 < indicates absence of hemagglutination at an antigen dilution of 1/2.

As can be seen from Table I, adult goose, adult chicken, and sheep RBC were agglutinated by the rubella virus preparations, but with less efficiency than the newly hatched chick RBC. Other RBC commonly used to demonstrate HA of myxoviruses (rhesus monkey, guinea pig, and human O) were not agglutinated.

**EXAMPLE II**

Unheated sera and gamma globulin samples for hemagglutination-inhibition (HAI) antibody assay were treated with kaolin and adsorbed with newly hatched chick RBC in order to remove non-specific inhibitors and agglutinins by the method described by Clarke et al. (see above).

The kaolin method suggested by Clarke et al. comprises preparing a 25 percent suspension of acid-washed kaolin by adding, with constant mechanical stirring, 25 g. of the powder to 100 ml of borate-saline, pH 9.0. So far as is known, this slurry keeps indefinitely at 4°C. To 1 volume of undiluted test serum are added 4 volumes of borate-saline and 5 volumes of 25 percent kaolin. The mixture is held at room temperature for 20 minutes with occasional shaking and is then centrifuged at 2,500 r.p.m., also at room temperature for 30 minutes. The supernatant fluid is at pH 9 and is considered to represent a 1:10 dilution; the inaccuracy introduced by not taking into account the volume occupied by the solid kaolin is disregarded. If, because of inadequate quantity, it is necessary to treat sera that are at higher initial dilutions than 1:5, this may be done by using correspondingly more dilute suspensions of kaolin.

The Clarke et al. method for removal of naturally occurring agglutinins for the erythrocytes from blood of the animal used in the hemagglutination inhibition comprises chilling the kaolin-treated sera in an ice bath to avoid the action of hemolysins, which also may be present in the sera. To each serum is added 0.1 ml of the packed erythrocytes per 5 ml of serum at the 1:10 dilution. Adsorption takes place for 20 minutes with occasional shaking, after which the tubes are centrifuged for 10 minutes at 1,500 r.p.m. in the cold. The supernatant fluids are used in the hemagglutination inhibition test.

Serial two-fold dilution of the specimens to be tested for antibody were mixed with equal volumes (0.025 ml) of antigens containing 4 HA units. The DGV–BPA buffer was used as diluent for all test components. After incubation of the serum-antigen mixtures for 15 minutes at 36°C, 0.025 ml of 0.16 percent chick RBC was added to each cup and the plates were then refrigerated for 1 to 1.5 hours. Antibody end points were read as the highest dilution of the sera or globulins producing complete inhibition of hemagglutination. Each test included an appropriate titration of antigen, positive and negative
control sera, serum-RBC, and diluent-RBC controls. The results are seen in Table 2.

| TABLE 2—INHIBITION OF RUBELLA VIRUS HEMAGGLUTINATION BY SPECIFIC ANIMAL ANTISERA |
|-------------------|-------------------|-------------------|-------------------|
|                   | HAI antibody titer |                  |                  |
| Virus             | Immunized host    | Homologous virus vs. Rubella virus |                  |
| Rubella           | Rabbit            | -                 | 512               |
|                   | Monkey            | -                 | 513               |
|                   | Guinea pig        | -                 | 42                |
| Rubella           | Horse             | 4,162             | <8                |
|                   | Monkey            | 4,056             | <8                |
| Mumps             | Guinea pig        | 212               | <8                |
|                   | Monkey            | 128               | <8                |
| Influenza         | A/FM1             | 800               | <40               |
|                   | A/PRA             | 1,400             | <15               |
|                   |                   | 1,600             | <10               |
| Parasites         | Guinea pig        | 252               | <8                |
|                   |                   | 125               | <8                |
|                   |                   | 255               | <8                |
| Respiratory syncytial |                   | 255               | <8                |
| Vaccinia          | Rabbit            | 1,025             | <8                |

1. Tested against 4 hemagglutinating units of antigen.
2. CF antibody titer.

As can be seen from Table 2 rubella virus hemagglutinin (4 units) was inhibited at high serum dilutions by specific rubella virus antisera from several sources. Antiserum from animals immunized with a number of myxoviruses, rubella, respiratory, syncytial, and vaccinia virus did not inhibit agglutination. Kaolin treatment of serum specimens was essential since all untreated human and animal sera tested contained non-specific inhibitors of rubella virus hemagglutination in 1:4 to 1:200 dilution. The kaolin treatment is well known and the aforementioned Clarke et al. article, the substance of which is incorporated herein in its entirety, may be referred to for further detail.

EXAMPLE III

The procedure of Example II was used to test specimens from persons infected with natural rubella and persons inoculated with the HPV-77 strain of attenuated rubella virus which was described in detail in the aforementioned copending application. This procedure was compared with conventional complement-fxation and neutralizing antibody test procedures referred to previously. The results are shown in Table 3.

| TABLE 3—HAI, CF AND NEUTRALIZING ANTIBODY IN NATURAL AND EXPERIMENTAL RUBELLA VIRUS INFECTIONS |
|----------------------------------------------------------|-------------------|-------------------|
| Serum source (rubella patients)                          | Antibody titer 1  |                  |
| Day serum collected | HAI | Neut. | CF |
| Children:                                                                                     |
| PW                                                     | 2-2             | <8              | <8 |
|                                                         | 22              | 1,024           | <8 |
|                                                         | 112             | 256             | NT  |
|                                                         | 38              | <8              | <8 |
|                                                         | 8               | <8              | <8 |
|                                                         | 1               | 5               | <8 |
|                                                         | 16              | 1,024           | 54  |
|                                                         | 106             | 256             | NT  |
|                                                         | 25              | 125             | 64  |
|                                                         | 113             | 256             | NT  |
| Military recruits:                                                                             |
| R-26                                                   | 1               | 5               | <8 |
|                                                         | 22              | 1,024           | 16  |
|                                                         | 38              | 1,024           | 16  |
|                                                         | 38              | 5               | <8 |
|                                                         | 1               | <8              | <8 |
| Adult:                                                  |
| JF                                                     | 3               | 125             | <8 |
|                                                         | 25              | 4,044           | 2   |
|                                                         | 4               | 256             | <8 |
|                                                         | 15              | 2,500           | <8 |
| Vaccinia (HPV-77):                                    |
| MY                                                     | 4               | <8              | <2 |
|                                                         | 42              | 256             | <8 |
|                                                         | 4               | <8              | <8 |
|                                                         | 42              | 256             | 8   |

1. Reciprocal of antibody titer when tested against 4 hemagglutinating antigen units, 4 complement fixing antigen units and 10 to 60 TCID₅₀ of rubella virus.
2. Day before or after onset of rash.
3. Not tested.
4. Day after inoculation.

Neutralizing and HAI antibodies appear early in the course of infection and can frequently be detected by the first day of rash in natural rubella. Maximum levels of these two types of antibody are attained by the 4th to the 8th week after infection. At any given period HAI antibody titers are at least eight-fold higher than the corresponding levels of neutralizing antibody. Both types of antibody appear with regularity during rubella virus infection; then when appropriately spaced, paired sera are available, either test can be used as a sensitive diagnostic tool, with the more sensitive HAI test being preferred. Complement fixing antibody tends to appear somewhat later during the convalescent phase of rubella but with less regularity. Three of the patients listed in Table 3 failed to develop CF antibody whereas all eight developed neutralizing and HAI antibodies. Persons infected with attenuated rubella virus rarely developed CF antibody but consistently showed neutralizing and HAI antibody responses.

EXAMPLE IV

Specimens from persons with rubella virus infection, and with measles, mumps, and vaccinia infections, were tested by the procedure of Example II and by HAI test procedures for the latter three infections. The results appear in Table 4.

| TABLE 4—SPECIFICITY OF HAI ANTIBODY RESPONSE IN PERSONS INFECTED WITH RUBELLA AND OTHER VIRUSES |
|----------------------------------------------------------|-------------------|-------------------|
| Source of serum                                          | Rubella | Measles | Mumps | Vaccinia |
| Children with rubella virus infection:                   |         |         |       |         |
| CV strain                                                 | <3     | NT     | <3    | NT      |
| CV strain                                                 | 2,056  | NT     | 2,056 | NT      |
| CV strain                                                 | <3     | NT     | <3    | NT      |
| CV strain                                                 | 512    | NT     | 512   | NT      |
| Children with measles virus infection:                   |         |         |       |         |
| AL strain                                                 | <3     | NT     | <3    | NT      |
| CV strain                                                 | 2,056  | NT     | 2,056 | NT      |
| CV strain                                                 | <3     | NT     | <3    | NT      |
| CV strain                                                 | 512    | NT     | 512   | NT      |
| Children with mumps virus infection:                     |         |         |       |         |
| CV strain                                                 | 512    | NT     | 512   | NT      |
| CV strain                                                 | 256    | NT     | 256   | NT      |
| Children with vaccinia virus infection (primary):         |         |         |       |         |
| AK strain                                                 | 8      | NT     | <3    | NT      |
| CV strain                                                 | 256    | NT     | 256   | NT      |
| CV strain                                                 | <3     | NT     | <3    | NT      |
| CV strain                                                 | <3     | NT     | <3    | NT      |

* Not tested.

As can be seen in Table 4, measles, mumps, and vaccinia infections neither led to the appearance of rubella HAI antibody nor altered existing levels of rubella antibody. Conversely, rubella virus infection had no effect on HAI tests for measles, mumps, or vaccinia antibodies.

EXAMPLE V

In this example the persistence of rubella virus HAI antibody was determined in comparison with neutralizing antibody and CF antibody. HAI antibody, like neutralizing antibody, apparently persists for many years. Both types of antibody were detected in each of 58 sera collected from adults 26-62 years old, unselected with respect to rubella virus history or exposure. Sera from 4 of these individuals with HAI antibody titers ranging from 1:64 to 1:2048 were also examined for CF antibody. All but one of these sera were devoid of CF activity.

EXAMPLE VI

Five lots of human gamma globulin contained rubella virus HAI antibody in titers ranging from 1:1024 to 1:4096. Neutralizing antibody titers on these samples were four to eight-fold lower, ranging 1:128 to 1:512. Since commercially prepared gamma globulins are prepared from plasma or placentas from numerous adult donors, these
results also suggest the persistence of rubella virus HA1 antibody and neutralizing antibody many years after infection.

Thus, the expensive, complicated, and time-consuming nature of the neutralization test will be readily recognized. Also, neutralizing antibody titers tend to be low in rubella, leading to difficulties in interpretation of results. Similarly, problems exist with the fluorescent antibody method. Neither test is particularly adapted for large scale use in diagnostic laboratories. As mentioned previously, while the complement fixation technique is relatively simple and provides results in 24 hours, the test is less sensitive than the neutralizing antibody test since rubella patients frequently fail to develop detectable CF antibody. Moreover, even when CF antibody appears, it is relatively transient. The CF method does not lend itself to epidemiological studies or to determining immunity in adults. Since attenuated rubella virus infections rarely evoke a CF antibody response, the test has no value in live vaccine studies.

On the other hand, the hemagglutination-inhibition test of the instant invention is quite sensitive, with antibody titers running about eight times higher than neutralizing or complement fixing antibody levels. Also, all cases develop HI antibodies, whereas not all persons with rubella became CF positive. Using the newly hatchet chick RBC's for hemagglutination it was shown that sera containing antibodies for rubella virus inhibit said hemagglutination, while antisera for other viruses do not.

It is therefore, readily apparent that the hemagglutination-inhibition test hereinabove disclosed and described lacks the disadvantages accompanying the other known serological tests for rubella.

Since there are modifications of the instant inventive concepts which will be obvious to those skilled in the art, all matter herein is to be considered illustrative and not in a limiting sense unless otherwise identified.

What is claimed is:

1. The method of serodagnosis of rubella by hemagglutination-inhibition comprising the steps of:
   (a) mixing a specimen to be tested, selected from the group consisting of blood serum and gamma globulin, with rubella virus antigen, to form a specimen-antigen mixture, each of said specimen and said antigen being free from non-specific inhibitors of hemagglutination;
   (b) adding erythrocytes obtained from a member selected from the group consisting of chicken blood, goose blood, and sheep blood, to form a specimen-antigen-erythrocyte mixture;
   (c) incubating said specimen-antigen-erythrocyte mixture for a period of time sufficient to allow settling;
   (d) wherein hemagglutination is inhibited when the rubella antibody is present in said test specimen.

2. The method of claim 1 wherein said chicken blood is obtained from the blood of chicks less than 24 hours old.

3. The method of claim 1 wherein the temperature of incubation is from about 4°C to about 37°C.

4. The method of claim 1 wherein each of said antigen and said erythrocytes are diluted with a diluent prior to mixing.

5. The method of claim 4 wherein said diluent comprises dextrose-gelatin-veronal buffer containing 0.2 percent bovine plasma albumin at pH 7.3.

6. The method of claim 5 further comprising serially two-fold diluting said specimen prior to mixing.

7. The method of claim 6 further comprising the steps of:
   (a) preparing said specimen by treating the same with kaolin and erythrocytes of said member prior to said serial two-fold dilution to remove non-specific inhibitors and agglutinins;
   (b) incubating said specimen-antigen mixture for from about 15 minutes to about 1 hour at room temperature;
   (c) suspending said erythrocytes in said diluent to a concentration of from about 0.16 percent to about 0.2 percent prior to adding said erythrocytes to said specimen-antigen mixture; and
   (d) incubating said specimen-antigen-erythrocyte mixture for from about 1 to about 1.4 hours at 4°C.

8. A method for detecting the presence of rubella antibodies in blood comprising the steps of:
   (a) preparing a specimen to be tested, selected from the group consisting of blood serum and gamma globulin by treating the same with kaolin and erythrocytes obtained from the blood of chicks less than 24 hours old to remove nonspecific inhibitors and agglutinins;
   (b) serially two-fold diluting said specimen with 0.025 mL volumes of a diluent comprising dextrose-gelatin-veronal buffer containing 0.2 percent bovine plasma albumin at pH 7.3;
   (c) diluting rubella virus antigen free of non-specific inhibitors of hemagglutination with said diluent to obtain 4 hemagglutination units of said antigen per 0.025 mL volume of antigen solution;
   (d) mixing 0.025 mL of said antigen solution, containing 4 hemagglutination units of said antigen, with each 0.025 mL volume of said specimen dilution, to form specimen-antigen mixtures;
   (e) incubating said mixtures for about 15 minutes at room temperature;
   (f) suspending erythrocytes obtained from the blood of chicks less than 24 hours old in said diluent to a concentration of about 0.16 percent;
   (g) adding 0.025 mL of said erythrocyte suspension to said specimen-antigen mixtures to form specimen-antigen-erythrocyte mixtures;
   (h) incubating said specimen-antigen-erythrocyte mixture for from about 1 to about 1.5 hours at about 4°C;
   (i) wherein hemagglutination is inhibited when the rubella antibody is present in said test specimen.

9. The method of claim 8 wherein said specimen is blood serum.

References Cited

UNITED STATES PATENTS


424--89: 195--1.1

OTHER REFERENCES


ALBERT T. MEYERS, Primary Examiner

A. P. FAGELSON, Assistant Examiner

U.S. Ct. X.R.