Modified Hemagglutination-Inhibition Test for
Rubella Employing Human Group O
Erythrocytes

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A hemagglutination-inhibition (HI) test for rubella is described which utilizes human group O, rather than 1-day-old chick, erythrocytes. The test was found to be as sensitive and reproducible for detection of rubella antibody as HI tests employing chick erythrocytes. Advantages to the use of human erythrocytes are (i) they are more available, (ii) it is unnecessary to absorb natural agglutinins from human test sera, and (iii) heparin-MnCl₂-treated sera do not agglutinate human erythrocytes, as is sometimes the case with chick erythrocytes. Factors influencing the reliability of the test are discussed.

The hemagglutination-inhibition (HI) test for rubella has found wide application in the diagnosis of rubella infections and in the determination of immunity status (3, 6, 8, 10, 19); for routine use, this test has largely replaced the more cumbersome and expensive neutralization and fluorescent-antibody tests.

Because of the important actions which may be taken by physicians on the basis of rubella HI test results, it is essential to use a technique of maximum sensitivity and reliability. Recognizing the need for standardization of the rubella HI test, the Center for Disease Control (CDC) appointed an ad hoc committee to develop a standard protocol for the performance of the test, based upon comparative studies between several laboratories (1). Major features of the CDC standard rubella HI test include standardization of the chick erythrocyte suspension by the cyanmethemoglobin method (9), the use of a N-2-hydroxyethylpiperazine-N′-2'-ethanesulfonic acid (HEPES) diluent (11), treatment of sera with heparin-MnCl₂ to remove nonspecific inhibitors (2, 12), and the use of 4 hemagglutinating (HA) units of antigen in the test.

It has been found in this laboratory that the use of a HEPES buffer system greatly extends the number of species of erythrocytes which are agglutinable by rubella virus (16), and advantage has been taken of this to modify the basic CDC rubella HI procedure to utilize human group O erythrocytes rather than 1-day-old chick erythrocytes.

This report describes the HI test procedure using human group O erythrocytes and compares results obtained in this test with those obtained with the CDC test or with the test procedure described by Halonen et al. (7).

MATERIALS AND METHODS

Sera examined. The modified HI test procedure was evaluated in tests on three groups of sera. These were (i) 289 sera which had been routinely submitted to this laboratory for serodiagnosis of rubella infection or for determination of immunity status, (ii) 42 coded sera from individuals vaccinated with Philips Roxane live attenuated rubella vaccine, HPV77-DK12, and (iii) a set of 92 coded, duplicate serum specimens which had previously been tested in one of the phases of the development of the CDC standard rubella HI test procedure.

Rubella HI tests using 1-day-old chick erythrocytes. Either the procedure described by Halonen et al. (7) using kaolin for absorption of nonspecific inhibitors from test sera or the CDC standard rubella HI test was employed in tests with chick erythrocytes. In some instances, sera treated with heparin-MnCl₂ were heated at 56°C for 15 min after treatment and before absorption with chicken erythrocytes to reduce the amount of nonspecific agglutination produced by the treated sera (N. Cremer, personal communication).

Rubella HI tests using human group O erythrocytes. The diluent used for the red blood cell (RBC) suspension, antigen, and test sera was the HEPES-saline-albumin-gelatin (HSAG) buffer described by Liebhaber (11). Tests were conducted by the Microtiter method using "V" plates (Linbro model IS-MVC-96, not processed).

Human group O RBC were collected into tubes
containing ethylenediaminetetraacetic acid (EDTA) anticoagulant and held at 4°C for 24 hr. The cells were washed three times in either physiological saline or dextrose-gelatin-Veronal (DGV) buffer by centrifuging at 900 × g for 10 min. They were then made into a 10% suspension in DGV; this suspension was used for up to 2 weeks. For use in the HI test, the RBC were standardized to a 4% suspension by using the cyanmethemoglobin method (9), and the 4% suspension (in DGV) was made into a 0.25% working suspension by dilution in the HSAG diluent. The working RBC suspension was not prepared in the HSAG until 15 to 30 min before it was to be used in the test.

Antigen was titrated in twofold dilutions in a volume of 0.025 ml in the presence of 0.025 ml of diluent and 0.05 ml of the RBC suspension. Tests were incubated at 4°C for 90 min, and 4 units of antigen, based upon complete agglutination, were employed in the HI test. The working dilution of antigen was not prepared in HSAG until 15 to 30 min before it was to be added to the test.

For removal of nonspecific inhibitors from the test sera, 0.1 ml of serum was mixed with 0.2 ml of HSAG and to this was added 0.1 ml of the 1:1 heparin-MnCl2 working solution (equal parts of heparin, 5,000 units per ml, and 1.0 m MnCl2). After mixing, the sera were held at 4°C for 30 min and then centrifuged at 900 × g for 15 min at room temperature. The supernatant fluid was carefully pipetted from the precipitate, and 0.2 ml was added to 0.2 ml of HSAG; this was considered to represent a 1:8 dilution of the test serum. It was unnecessary to absorb the sera with RBC.

Serial twofold dilutions of each serum from 1:8 through 1:2,048 were tested in a volume of 0.025 ml against a dilution of antigen containing 4 HA units in a volume of 0.025 ml. Serum-antigen mixtures were held at 4°C for 1 hr, and 0.05 ml of the 0.25% RBC suspension was added. Each test included the appropriate controls (1). Tests were incubated at 4°C for 90 min, and then held for 30 min at room temperature. The HI antibody titer was the highest dilution showing complete inhibition of HA.

Rubella virus neutralizing antibody assays. In certain instances, the results obtained in HI tests were confirmed by neutralization tests. These were conducted by a micro-metabolic inhibition technique developed in this laboratory. Tests were performed in disposable plastic plates (Linbro model IS-MRC-96) which were soaked in 95% ethyl alcohol for 2 hr, rinsed in distilled water, and, sterilized by ultraviolet irradiation before use. Cells of the RK-13 rabbit kidney line were employed. The cell suspension, containing 100,000 cells per ml, was prepared in a medium consisting of 95% Eagle's minimal essential medium (MEM, prepared in Hanks balanced salt solution containing 0.88 g NaHCO₃ per liter) and 5% inactivated fetal bovine serum. The serum and virus dilutions were prepared in the same medium supplemented with unactivated horse serum at a concentration of 10%. Test sera were inactivated at 56°C for 30 min, and serial dilutions were prepared in a volume of 0.05 ml. Each serum dilution was tested against two concentrations of virus [10 and 32 median tissue culture infective doses (TCID₅₀)] in a volume of 0.05 ml. Test virus preparations were prepared in BHK-21 cells, and they had titers of at least 10⁴ TCID₅₀ per ml based upon a colorimetric end point. Serum-virus mixtures were incubated at 37°C for 1 hr and then 0.05 ml of the cell suspension (5,000 cells) was added to each cup. The cups were sealed with 0.08 ml of sterile mineral oil (viscosity 350). Tests were incubated at 35°C for 4 days to permit optimal multiplication of the rubella virus, and then at 37°C for an additional 3 to 4 days to permit greater cellular metabolism with the production of acid. This incubation schedule resulted in sharper colorimetric end points for both virus and antibody titrations than were obtained with a constant temperature. A pH of 7.4 or higher, as indicated by the phenol red indicator, was considered indicative of a viral cytopathic effect or lack of neutralization, whereas a pH of 7.2 or lower was taken as evidence of virus neutralization.

RESULTS

Agglutination of human group O erythrocytes by various rubella antigens. Table 1 compares the HA titer of some representative rubella antigens against human group O RBC and against 1-day-old chick RBC. It was found that only antigens which had been treated with Tween 80 and ether (15) agglutinated human RBC, and, in some instances, two successive

| Antigen tested | HA titer against RBC* |
|----------------|-----------------------|
|                | Human O | 1-Day-old chick |
| Infected BHK-21 fluids concn 10X | <8 | 32 |
| Infected BHK-21 fluids concn 10X, T-E 1X | 32 | 128 |
| Infected BHK-21 fluids concn 10X, T-E 2X | 64 | 256 |
| Infected BHK-21 fluids concn 20X, T-E 1X | 128 | 256 |
| Alkaline extract of infected BHK-21 cells, T-E 1X | 128 | 512 |
| Microbiological Associates lot no. 3-3631 | 16 | 32 |
| Flow Laboratories lot no. C961151 | 4 | 32 |
| Flow Laboratories lot no. C961231 | 64 | 256 |
| Courtland Laboratories lot no. K690728 | 16 | 128 |
| Courtland Laboratories lot no. K701203 | 16 | 64 |
| Grand Island Biological Co. lot no. 078 | 32 | 128 |
| Grand Island Biological Co. lot no. 101 | 64 | 256 |

* HA, hemagglutinating; RBC, red blood cells.

* Treated with Tween 80 and ether one time.
Tween-ether treatments have resulted in an increased antigen titer for human O cells. Antigen titers tended to be fourfold lower against human RBC than against chick RBC, but some antigens gave only twofold lower titers. Certain antigens from commercial sources gave satisfactory HA titers with human RBC, and others did not. The methods used for preparation of these commercial antigens were not known.

Comparison of rubella HI antibody titers obtained in tests with human group O erythrocytes and with 1-day-old chick erythrocytes. Table 2 compares the rubella HI antibody titers obtained on 289 diagnostic serum specimens in tests using the two species of erythrocytes. Tests with chick RBC were conducted by the CDC standard procedure. (Approximately one-half of the sera were heated after heparin-MnCl₂ treatment, as indicated above, to reduce nonspecific agglutination by the sera.) Tests with human O erythrocytes were conducted as indicated above. The only differences between the two procedures were in the RBC employed, the higher concentration of antigen used for tests with human RBC, the omission of absorption with RBC for tests with human cells, and the heat treatment indicated above for certain sera tested against chick RBC.

Despite the fact that more antigen was employed in tests with human O RBC, antibody titers tended to be higher than those obtained with 1-day-old chick RBC. Approximately 61% of the sera showed the same HI titers with both types of RBC, 27% had twofold higher titers with human RBC, and 7% had fourfold higher titers. On the other hand, only 5% of the sera had higher titers in tests with 1-day-old chick RBC. A few sera showed low titers of 1:8 with human RBC, but titers of <1:8 with chick RBC. The specificity of the inhibition reactions of these sera was confirmed by the fact that the sera showed neutralizing antibody titers for rubella virus in the range of 1:8 to 1:32.

Generally, antibody levels elicited by attenuated rubella vaccines are lower than those produced by natural infections (4, 5, 13, 14), and it has been demonstrated that different rubella HA antigens may vary in their sensitivity for detecting vaccine-induced HI antibody (17). Since one of the most important uses of the rubella HI test is for determination of immunity status, it was of interest to determine the sensitivity of the HI test with human RBC for demonstration of HI antibody in vaccines. Pre- and postvaccination sera from 21 individuals who had received the Philips Roxane HPV77-DK12 vaccine were tested; these sera had previously been examined by the method of Halonen et al. (7) with 1-day-old chick RBC and kaolin absorption for removal of inhibitors. Table 3 shows that the HI

| HI titers vs. 1-day-old chick erythrocytes | HI titers vs. human group O erythrocytes |
|-------------------------------------------|-----------------------------------------|
| <8 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | ≥ 1,024 | Totals |
| ≥ 1,024 | 5 | 5 | | | | | | | |
| 512 | | | | | | | | | |
| 256 | 1 | 15 | 12 | 3 | 31 | | | | |
| 128 | 7 | 11 | 11 | 29 | | | | | |
| 64 | 4 | 22 | 12 | 5 | 43 | | | | |
| 32 | 2 | 19 | 16 | 8 | 45 | | | | |
| 16 | 11 | 14 | 5 | 30 | | | | | |
| 8 | 2 | 3 | 1 | 6 | | | | | |
| <8 | 86 | 6 | | | | | | | |
| Totals | 86 | 8 | 16 | 38 | 51 | 31 | 32 | 17 | 10 | 289 |

* Hemagglutination-inhibition. RBC, red blood cells.

Table 3. Postvaccination rubella antibody titers of individuals receiving attenuated virus vaccine

| Test system* | No. of postvaccination sera† with titers of |
|--------------|-----------------------------------------|
| HI, chick RBC, antigen C961231 | 7 | 11 | 3 |
| HI, human RBC, antigen 589-90 | 3 | 6 | 12 |
| HI, chick RBC, antigen 525 | 8 | 7 | 6 |
| Neutralization | 1 | 3 | 7 | 5 | 1 |

* Hemagglutination-inhibition. RBC, red blood cells.

† Prevaccination titers were <1:8 in all test systems.
titers obtained on the postvaccination sera with human group O cells were of approximately the same order as those obtained with chick RBC and an antigen shown to be highly sensitive for detection of rubella antibody (17, 18). Titers with human O cells were considerably higher than those obtained with chick RBC and an antigen which was relatively insensitive for detection of rubella antibody (17, 18). None of the prevaccination sera showed HI activity by any technique.

**Reproducibility of rubella HI antibody titers in tests with human group O erythrocytes.** Ninety-two serum specimens representing 46 duplicate, coded samples were examined in HI tests with human RBC. These had previously been tested by the Halonen procedure (7) with 1-day-old chick RBC as one phase of the reproducibility studies for the development of the CDC standard test procedure. Reproducibility consisted of the titers of the duplicate samples varying by no more than twofold. Table 4 shows that titers obtained with human RBC were reproduced with 100% of the specimens. Tests with chick RBC using the Halonen method showed a reproducibility of 97.3%. Sera were tested “blind” by both techniques.

**DISCUSSION**

Rubella HI tests employing human group O erythrocytes as indicator cells showed a high degree of sensitivity and reproducibility in the detection of antibody. Despite the fact that more antigen was required for tests with human RBC, this did not result in lower antibody titers, as has been observed in tests with goose erythrocytes (18), which also require more antigen than do chick RBC. In fact, there was a tendency for antibody titers to be somewhat higher in tests using human RBC.

Some laboratories using heparin-MnCl₂ (or dextran sulfate-CaCl₂) treated sera in HI tests with chick RBC have experienced difficulty with the lower serum dilutions producing agglutination of the RBC; this may mask low levels of antibody. With human group O erythrocytes this problem has not occurred, and thus a more reliable method than kaolin absorption can be employed for removal of nonspecific inhibitors from test sera. Kaolin may absorb immunoglobulin (Ig) M antibody, different batches of kaolin may vary in their ability to remove nonspecific inhibitors, and, further, antibody titers of kaolin-treated sera show greater variation from run to run than do those of sera treated with heparin-MnCl₂.

Rubella HI tests with human RBC are also advantageous from the standpoint of utilizing a more readily available species of erythrocytes and eliminating the need for routine absorption of human test sera with RBC to remove natural agglutinins. An occasional serum specimen may contain cold agglutinins for human RBC, but these can be absorbed out, if necessary, to obtain an antibody end point. A practical consideration for smaller laboratories is that, since it is unnecessary to absorb test sera routinely with RBC, a refrigerated centrifuge is not essential for rubella HI testing.

Certain minor details of the test procedure were found to affect the accuracy and clarity of results. In processed Linbro “V” plates, the test erythrocytes settled too slowly, and, therefore, unprocessed plates were employed. More clear-cut antibody titers were obtained by treating the sera with heparin-MnCl₂ at a serum dilution of 1:4, removing this from the precipitate, and then diluting to 1:8, rather than diluting the treated serum to 1:8 before removing it from the precipitate. It was found preferable to prepare the working suspension of erythrocytes and the working dilution of antigen in the HSAG buffer 15 to 30 min before addition to the test. RBC suspensions held for prolonged periods at pH 6.2 occasionally showed slightly rough settling patterns, and antigens held at pH 6.2 occasionally showed a slight decrease in titer.

The HSAG diluent was satisfactory for rubella HI tests with human RBC, but, if serum controls were carried through as many dilutions as the test proper, it was noted that RBC in the higher serum dilutions formed a very faint halo of cells around the compact button of unagglutinated RBC, ostensibly due to the lower protein concentration in the higher serum dilutions. This did not occur in the test proper, as the antigen apparently provided additional protein. This halo effect could be

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**Table 4. Reproducibility of rubella hemagglutination-inhibition tests conducted with human group O erythrocytes**

| Titters of duplicate specimens | No. of duplicate specimens |
|--------------------------------|-----------------------------|
|                                | Human group O RBC* | 1-Day-old chick RBC* |
| Same titters                    | 31                  | 30                      |
| Twofold difference             | 15                  | 15                      |
| Fourfold difference            | 0                   | 1                       |
| Reproducibility                | 100%                | 97.3%                   |

* Red blood cells.
* Test conducted by the method of Halonen et al. (7) using kaolin absorption for removal of inhibitors.
abolished by using a modified diluent containing 0.14 M NaCl, 0.025 M HEPES, 1.5 g of bovine albumin per liter, 15 mg of gelatin per liter, and 0.015 M CaCl₂. The higher concentration of Ca²⁺ also resulted in slightly higher antigen titers.

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