Compatibility of Trypsin-Modified Human Erythrocytes in the Rubella Hemagglutination-Inhibition Test Employing Three Serum Treatment Procedures

DONALD B. NELSON, EDWARD P. QUIRIN, AND STANLEY L. INHORN
Department of Preventive Medicine and Wisconsin State Laboratory of Hygiene, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 23 November 1973

Results of comparative tests using trypsin-modified human type O erythrocytes and cells from newly hatched chickens with three standard serum treatment methods for rubella hemagglutination-inhibition techniques are reported. The kaolin, heparin-manganous chloride and dextran sulfate-calcium chloride methods could all be used with both cell types. Reproducibility with heparin-manganous chloride and dextran sulfate-calcium chloride was excellent with both cell types. Both methods gave generally higher antibody titers than the kaolin procedure. However, the use of human cells resulted in a more sensitive system than chicken cells with all serum treatment methods.

In a recent publication (4), we reported the use of trypsin-modified human erythrocytes in the rubella hemagglutination (HAI) and hemagglutination-inhibition (HI) tests. Treated human erythrocytes were found to be as sensitive to agglutination by a variety of rubella antigens as fresh 0- to 2-day-old chicken cells. With test sera treated by dextran sulfate-calcium chloride for the removal of nonspecific inhibitors (3), the human cell indicator serum was at least as sensitive as newly hatched chicken cells in demonstrating inhibition of agglutination by specific antibody. Relatively few laboratories use the dextran sulfate-calcium chloride serum treatment method. A number of inquiries have been received concerning the adaptability to and efficiency of trypsin-treated human erythrocytes with the kaolin and the heparin-manganous chloride serum treatments. This study was undertaken to compare results of these three serum treatment methods by using both human cells and the more commonly used chicken cells.

MATERIALS AND METHODS

Test sera. Thirty-two sera were selected from specimens received for routine rubella serology that had been tested by our dextran sulfate-calcium chloride method. The only criteria employed in their selection were that sufficient volume be available for multiple testing and that a wide range of titers be represented.

To eliminate any possible bias, a blind study procedure was instituted. The 32 sera were given to a member of our staff with instructions to code them into six groups of 48 specimens each so that duplicates and other multiples would be included in each group. Reproducibility within any given serum treatment-cell type system then could be tested. The technician performing the tests had no knowledge of the titers to be expected in any case. The sera were distributed into numbered vials in 0.2-ml samples so that testing could be done simply by adding the prescribed reagents.

Serum treatment. Testing was done on four separate days with 12 sera processed by each of the three serum treatment methods and tested with each red cell type (human "O" and chicken) for a total of 288 tests. The kaolin procedure was performed with reagents and the recommended procedure from Flow Laboratories, Rockville, Md., except that the reagent volumes were modified so that serum dilutions began at 1:8 rather than 1:10 in order to make the test results comparable by all methods. Reagents for the heparin- and dextran-sulfate procedures were made in this laboratory as previously described (1). Lipohepin (Riker Laboratories), 5,000 units/ml, and dextran sulfate (5% aqueous solution) were used in these respective procedures. The heparin procedure was that recommended by the Center for Disease Control (CDC) (1) and the dextran-sulfate method was our modification of Liebhaber's procedure (2), previously reported (3). Absorption of sera for testing with chicken cells was done by addition of 0.1 ml of 10% cells as previously reported (3). Sera tested with human cells were not absorbed.

Antigen and erythrocyte preparation. Antigen used in all tests was produced in this laboratory in Vero tissue culture (derived from African green monkey [Cercopithecus aethiops] kidney) by the method of Liebhaber (2) and four HA units were used in all tests. All tests were done in microtitration "V" plates (Linbro Chemical Co.). Cells from chickens
were collected in Alsever’s solution within 2 days of hatching, and were washed immediately in dextrose-gelatin-Veronal (DGV) buffer. Human cells from a volunteer were collected and stored at 4°C in Alsever’s solution until trypsinization. The trypsin used was lyophilized (beef pancreas), 100 mg/ampoule, and was reconstituted by the addition of 10 ml of distilled water. The human cells were washed in DGV, a 10% suspension was prepared, and 0.1 ml of the trypsin solution was added for each milliliter of cells. The suspension was mixed by gentle inversion of the tube and was incubated at room temperature for 1 h. The cells were then rewarshed in DGV and stored at 4°C as a 10% stock concentration in DGV. Cells stored in this manner could be used for 5 to 7 days after treatment. For use in the test, a further 1:40 dilution of this stock suspension was made either in red blood cell diluent (kaolin procedure) or N-2-hydroxyethylpiperazine-N’-2’-ethane-sulfonic acid-saline-albumin-gelatin (HSAG) (dextran sulfate or heparin procedure) to give a final concentration of 0.25%.

Preparations using chicken cells were incubated at 4°C for 1.5 h and those using human erythrocytes were incubated at 37°C for 1 h. Previous testing (4) has shown that human cells agglutination pattern to be most easily read at 37°C, but no difference in levels of sensitivity is detectible between 4 and 37°C. In all test variations, the end points were confirmed by placing the plates on their sides and observing the streaking or running of the non-agglutinated cells.

**RESULTS**

Agglutination patterns were essentially stable with both cell types used. On occasion, negative sera (<1:8) showed some breakdown of the agglutination pattern at the lower serum dilutions (1:8 and 1:16). In these few cases the cells formed rough buttons which could be easily distinguished from specific inhibition by “running” of the buttons when the plates were held vertical on their sides for 15 to 30 s.

A summary of all test results is shown in Table 1. Complete agreement was seen with the negative sera; all 10 showed titers of <1:8 by all serum treatment procedures and with both cell types. The end point with both cell types was usually seen when testing was made at 37°C. At 4°C, the end point was sometimes negative. Sensitivity recordings sometimes showed scantly agglutination at 4°C and an essentially stable pattern at 37°C.

### Table 1. Rubella HAI titers determined by use of trypsinized human cells and newly hatched chicken cells

| Specimen no. | Human cells | Chicken cells | Range of titers |
|--------------|-------------|---------------|----------------|
|              | Test method | Test method   |                |
|              | Kaolin      | Dextran       | Heparin-MnCl₂ | Kaolin | Dextran | Heparin-MnCl₂ |            |
| 5609, 5570  | <8<8        | <8<8          | <8<8<8        | <8     | <8<8    | <8<8<8       | <8-16     |
| 1705, 2426  | 16 8        | 16 8          | 8<8<8<8       | 8      | 16<8    | 16<8<8       | <8-16     |
| 1551, 2447  | 16 32       | 64 64         | 8<8<8<8       | 8      | 16<8    | 16<8<8       | 8-64      |
| 671, 2237   | 32 32       | 48<48*        | 16<8<8<8      | 16     | 16      | 16<8<8       | 8-48      |
| 174, 2040   | 32 48       | 102<102       | 48<8<8<8      | 24     | 24<8    | 24<8<8       | 128-24     |
| 493         | 16 8<8      | 48<8<8       | 8<8<8<8<8     | 16     | 16<8    | 16<8<8       | 128-16     |
| 1965        | 64 128      | 64<128<128    | 64<8<8<8<8    | 64     | 64      | 64<8<8<8     | 128-32     |
| 1971        | 128 64      | 128<128<128  | 64<8<8<8<8<8 | 64     | 64      | 64<8<8<8<8<8 | 128-32     |
| 2017        | 64 128      | 128<128<128  | 64<8<8<8<8<8 | 64     | 64      | 64<8<8<8<8<8 | 128-32     |
| 1365        | 48 128      | 64<128<128   | 64<8<8<8<8<8 | 64     | 64      | 64<8<8<8<8<8 | 128-32     |
| 1077        | 96 192      | 192<192<192  | 32<8<8<8<8<8 | 32     | 96      | 96<8<8<8<8<8 | 128-32     |
| 1268        | 96 96       | 96<96<96     | 32<8<8<8<8<8 | 32     | 48      | 48<8<8<8<8<8 | 128-32     |
| 2080        | 64 128      | 192<192<192  | 48<8<8<8<8<8 | 48     | 64      | 64<8<8<8<8<8 | 128-32     |
| 1058        | 96 128      | 256<256<256  | 32<8<8<8<8<8 | 32     | 48      | 48<8<8<8<8<8 | 128-32     |
| 1065        | 128 256     | 384<384<384  | 64<8<8<8<8<8 | 64     | 128     | 128<8<8<8<8<8 | 128-32     |
| 1133        | 256 384     | 512<512<512  | 128<256<256<256 | 128     | 128     | 128<8<8<8<8<8<8 | 128-32     |
| 2135        | 192 512     | 384<384<384  | 128<256<256<256 | 128     | 128     | 128<8<8<8<8<8<8<8 | 128-32     |
| 2134        | 256 512     | 512<512<512  | 128<256<256<256 | 128     | 128     | 128<8<8<8<8<8<8<8<8 | 128-32     |

*Reciprocal of HAI titer.

*Titers of 24, 48, 96, 192, and 384 result from interpolation of differing results with duplicate specimens.
types. Of the remaining 22 sera, all showed predominantly higher titers with the human cells than with chicken cells. Most sera also showed higher titers with dextran-sulfate or heparin treatment when compared with kaolin treatment in the same cell system. The geometric mean titer of all positive tests run by using human cells was 94.9, whereas that of the tests using chicken cells was 50.0 (Table 2). With each of the three serum treatment methods, the geometric mean titers with human cells were considerably greater than those determined with chicken cells. Although none of the sera treated with dextran sulfate or heparin varied in titer by more than one twofold dilution within each red cell system (Table 1), the geometric means indicated slightly higher values for the heparin procedure regardless of the cell used.

Comparing titers obtained by using the different serum treatment methods within the two indicator cell systems (Table 3) shows that, using human cells, 17 sera treated with dextran sulfate and 18 treated with heparin showed higher titers than those treated with kaolin; kaolin-treated sera had higher titers than dextran-sulfate-treated sera in two cases and in no case when compared with heparin-treated sera. A similar relationship, but less pronounced, is shown when comparison is made of the three serum treatment methods using chicken cells as the indicator. Comparison of the results with dextran sulfate and heparin show no more than twofold variation in titers within each cell system. However, within each cell system, the dextran-sulfate and heparin methods showed a consistently higher range of titers than the kaolin treatment.

Table 4 compares titer differences by indicator cells with the different serum treatment methods. Human cells gave higher values than chicken cells in 19 of 32 tests with kaolin, 21 of 32 with dextran sulfate, and 22 of 32 with heparin. No sera showed a higher titer with chicken than with human cells, regardless of serum treatment method.

A total of 19 sera were tested in duplicate for reproducibility in one or more of the systems; results are shown in Table 5. Of the 84 tests only five show a fourfold difference; the remaining 79 tests (94%) show no more than the twofold difference that is commonly considered within normal variation, even though none of the repeat testing was done on the same day in the same test system. The duplicate tests were done on sera ranging in titer from 1:8 to 1:512. There appears to be no significant difference in the reproducibility of results obtained within the various serum treatment-cell system combinations.

**DISCUSSION**

The use of trypsin-modified human erythrocytes in the rubella HAI test offers several distinct advantages over the presently recommended chicken cells. The availability of human cells to laboratories without reliance upon commercial sources, is of practical impor-
tance. Particularly for the small laboratory, the use of such cells can offer a significant saving over commercial cells.

Aside from these advantages it appears warranted to advocate the use of human indicator cells on the basis of efficacy alone. Trypsinized human erythrocytes are more sensitive than chicken cells. Quite frequently a two- to fourfold higher titer is demonstrated with human cells than with chicken cells. In this study, as well as in our routine use, there is no indication that this increased sensitivity is reflected in an increased number of false positives. Reproducibility of serum antibody titers is very good with human cells from a variety of type O donors, either Rh positive or Rh negative, providing the serum treatment method used is constant. These data also show that the trypsin-treated human cells are compatible with all three serum treatment methods used for removal of nonspecific inhibitors. We have shown in previous work that trypsin-modified cells can be used for a week or more with little or no loss of sensitivity to rubella antigens (4). Others have presented work with untreated human O cells which usually results in antigen titers two- to eightfold lower than with chicken cells (5). Although their serum titers were shown to be similar to, or higher than, those obtained with chicken cells, more concentrated antigen was required. This is both more costly and impractical, since many commercial antigens have titers in the range of 1:32 to 1:64.

Two minor changes have been made in our technique as originally reported (4). The cells are now washed in DGV both prior to and after trypsinization and the working cell concentration has been changed from 0.3% to 0.25%. Both changes tend to give higher antigen titers and the use of DGV results in more stable cells which are usable for a longer period following trypsinization.

When the heparin-MnCl₂ and the dextran-sulfate-CaCl₂ serum treatments were recommended by CDC in 1970 (1), we tried both methods. With chicken cells we have had limited success with the heparin technique, because of the development of nonspecific hemagglutinins in treated sera. The dextran-sulfate method was adopted although we have on occasion seen the same difficulty with nonspecific agglutinins at the lower serum dilutions. Several private laboratories in this state have attempted to use both of the newer serum treatment techniques with chicken cells, but have returned to the kaolin procedure because of the problem with nonspecific agglutination. This problem did not occur with the trypsinized human cells with either serum treatment.

In this study we have shown that all of the three serum treatment procedures currently available may be used with trypsin-modified human cells. The dextran-sulfate and heparin methods are preferred to kaolin since both give a specific precipitation of the beta-lipoprotein fraction of serum which contains the nonspecific inhibitor to rubella HAI. In addition, both techniques give higher antibody titers than kaolin treatment with both chicken and human cells. Using trypsin-modified human cells with either the heparin-MnCl₂ or dextran-sulfate-CaCl₂ serum treatment methods will hopefully contribute to greater standardization, sensitivity, and reproducibility of the rubella HAI test as applicable to the diagnostic laboratory.

**LITERATURE CITED**

1. CDC standard rubella hemagglutination-inhibition test. 1970. Center for Disease Control, U.S. Public Health Service, Washington, D.C.
2. Liebhaber, H., T. Pajot, and J. T. Riordan. 1969. Growth of high titered rubella virus in roller bottle cultures of VERO cells. Proc. Soc. Exp. Biol. Med. 130:12-14.
3. Nelson, D. B., E. P. Quirin, and S. L. Inhorn. 1972. Improved dextran sulfate-calcium chloride method for the removal of non-specific inhibitors with modifications for non-specific agglutinin removal in the rubella hemagglutination-inhibition test. Appl. Microbiol. 24:384-389.
4. Quirin, E. P., D. B. Nelson, and S. L. Inhorn. 1972. Use of trypsin-modified human erythrocytes in rubella hemagglutination-inhibition testing. Appl. Microbiol. 24:383-387.
5. Schmidt, N. J., and J. Dennis. 1972. Modified hemagglutination-inhibition test for rubella employing human group O erythrocytes. Appl. Microbiol. 23:471-475.